Regulation of T cell responses in atherosclerosis

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Chapter 1

General introduction

Atherosclerosis

Atherosclerosis is a chronic lipid-driven inflammatory disease affecting arterial blood vessels. The onset of atherosclerosis already starts in the second decade of life and persists. Traditionally, atherosclerosis was seen as a lipid disorder in which the vascular wall became filled up with lipids, but nowadays it is widely accepted that inflammation plays an important role during atherosclerotic lesion formation. It is considered to be an enemy of human mankind and per year many people in the Western world die from cardiovascular events. In 2006, 33% (45.445) of all people that died that year in the Netherlands, died from a cardiovascular event. High fat intake, smoking, sedentary life-style and stress are only a few examples of factors that increase the risk for atherosclerosis. Besides these risk factors, other disorders of the body such as diabetes, dyslipidemia and obesity correlate with the incidence of atherosclerosis. The current therapies for atherosclerosis are mainly directed to these risk factors. The most prescribed medicines aimed at lowering plasma cholesterol levels are statins. Together with better life style advices, this has resulted in a strong decline in the incidence of atherosclerosis, but cardiovascular disease (CVD) is still the leading cause of death in Western societies. Therefore, additional therapies to treat atherosclerosis are highly relevant and will be beneficial in the further lowering of CVD.

Lesion development

Initial lesion formation

Atherosclerosis normally starts during adolescence in most major medium and large sized arteries and is in the beginning asymptomatic. Atherosclerosis starts with a dysfunctional endothelium at predisposed sites. A normal healthy endothelium plays an important role in maintaining vessel wall homeostasis and protects against the adhesion of platelets and inflammatory cells, thrombus formation and the proliferation of vascular cells.[1] Dysfunction of the endothelium can be induced by turbulent or oscillatory shear stress, smoking, hypertension, obesity, atherogenic lipoproteins such as very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) and possibly by infectious microorganisms such as *Chlamydia pneumoniae*. These factors induce an increased permeability of the endothelial cell layer for lipoproteins and elevated expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1),

intercellular adhesion molecule-1 (ICAM-1), E-selectin and P-selectin.[2,3] Endothelial cells also start producing chemokines (monocyte chemotactic protein-1 (MCP-1)), growth factors and vasoactive molecules which affect the blood pressure.[4] Both the deposition of lipids and the expression of the adhesion molecules correspond with the location where a fatty streak, the first phase of the atherosclerotic plaque, is formed.[5] The adhesion molecules are responsible for rolling of leukocytes on, and subsequent adhesion to, the endothelium. The leukocytes, monocytes or T cells, subsequently migrate through the endothelial layer into the intima (Figure 1.1). Under influence of different cytokines such as tumor necrosis factor-α (TNFα), macrophage colony stimulating factor (M-CSF), interferon-γ (IFN-γ), interleukin (IL)-1β, IL-2 and transforming growth factor-β (TGF-β), monocytes differentiate into macrophages.[6,7] T cells can undergo antigen-dependent activation within the intima. Experiments using genetically altered mice show the importance of the adhesion molecules. E-selectin and P-selectin deficiency in apolipoprotein E deficient (apoE$^{-/-}$) mice results in a reduced severity of atherosclerosis.[8] LDL receptor deficient (LDLr$^{-/-}$) mice depleted for functional VCAM-1 also show reduced plaque formation.[9] Besides adhesion molecules, also chemokines contribute to the recruitment of the leukocytes into the vessel wall. Depletion of chemokine (CC) ligand 2 (CCL2) and its receptor CCR2 reduce atherosclerosis[10,11], while also CCL5 (RANTES), chemokine (CXC) ligand 10 (CXCL10) and CXCL11 are produced within the atherosclerotic plaque.[12] Blocking CXCR3 reduces the migration of effector T cells into the vessel wall and consequently results in an attenuation of atherosclerotic lesion formation (van Wanrooij et al., submitted for publication). All these data confirm the importance of the immune system in the early stages of atherosclerosis.

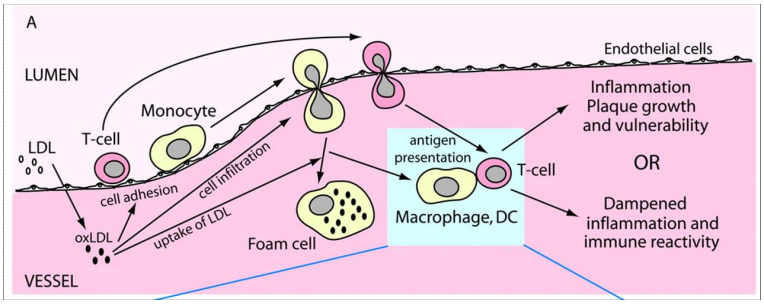

Figure 1.1: Effects of oxidized LDL on leukocyte recruitment into the vessel wall. LDL taken up in the vessel wall may get oxidized. The oxidized LDL stimulates the adhesion and infiltration of both T cells and monocytes into the vessel wall. The monocytes differentiate into macrophages and take up oxLDL which results in foam cell formation. T cells may be activated after presentation of the antigen by macrophages or DCs. This activation may result in inflammation and subsequently in plaque growth or it can result in a dampened inflammatory status (adapted from Robertson and Hansson) [13]

The other important contributor to the development of atherosclerosis is the disturbed lipid metabolism. Due to the increased permeability of the vessel wall, lipids and especially LDL-cholesterol accumulate in the vessel wall. The cholesterol is incorporated in the monolayer surrounding the lipoproteins which

further consist of phospholipids and apolipoproteins. The core of the lipoprotein is filled with cholesteryl esters and triglycerides. The lipoprotein family can be divided in chylomicrons, VLDL, intermediate-density lipoprotein (IDL), LDL and high-density lipoprotein (HDL).[14] The two most important lipoproteins are LDL, often seen as "bad cholesterol" and HDL also known as "good cholesterol". Both lipoproteins carry cholesterol in the blood stream. HDL transports cholesterol from the periphery to the liver where it is excreted from the body via the bile, while LDL transports the cholesterol from the liver and intestines to several tissues.
LDL is also entrapped within the vascular wall. Within the intima, the accumulated LDL may undergo modification by oxidation, glycation, aggregation, association with proteoglycans or incorporation into immune complexes.[15–17] Oxidized LDL (oxLDL) can be internalized by the tissue macrophages via scavenger receptors.[15,17–19] This internalization of oxLDL leads to the formation of lipid peroxides and facilitates the accumulation of cholesterol esters, because cholesterol derived from oxLDL cannot be mobilized from the cell sufficiently. This results in the formation of lipid-rich foam cells. The modified LDL is also chemotactic for other monocytes, attracting more and more of them into the intima. This may expand the inflammatory response and now also more T cells are attracted into the intima (Figure 1.1). In this stage, the lesion is called a "fatty streak" and remains asymptomatic.

Progression of lesions

Once smooth muscle cells start to migrate from the media into the lesion a progressive lesion is formed. The smooth muscle cells start to cover the fatty streak and extracellular matrix components are produced,[20] resulting in a fibrous cap that covers a necrotic core which is composed of cell-free lipids and cholesterol crystals. The lesion is now called an advanced atherosclerotic lesion and grows into the lumen of the vessel.[21,22] Consequently, the lesion is exposed to mechanical forces of the blood stream (shear stress) and the lesion is getting increasingly instable. Instability is also caused by other factors such as IFN-γ produced by T helper 1 (Th1) cells. IFN-γ inhibits both the proliferation of vascular smooth muscle cells and the production of collagen by these cells.[23,24] In addition, macrophages are very important in reducing plaque stability via the production of matrix-degrading proteases such as matrix metalloproteinase (MMP)-1, MMP-8, MMP-9 and MMP-13.[25] Also the presence of a number of immune cells and especially dendritic cells (DCs) in the shoulder regions of the plaque increase the vulnerability.[26] The advanced lesions may become symptomatic when the fibrous cap ruptures and the necrotic core is exposed to the bloodstream.[27] This induces thrombus formation which may occlude arteries in various organs and lead to lethal myocardial infarction or strokes and also in acute limb ischemia. Enhanced instability of the plaque increases the chance of a rupture (Figure 1.2). From all these data it can be concluded that the immune system is also accelerating the disease in advanced stages.

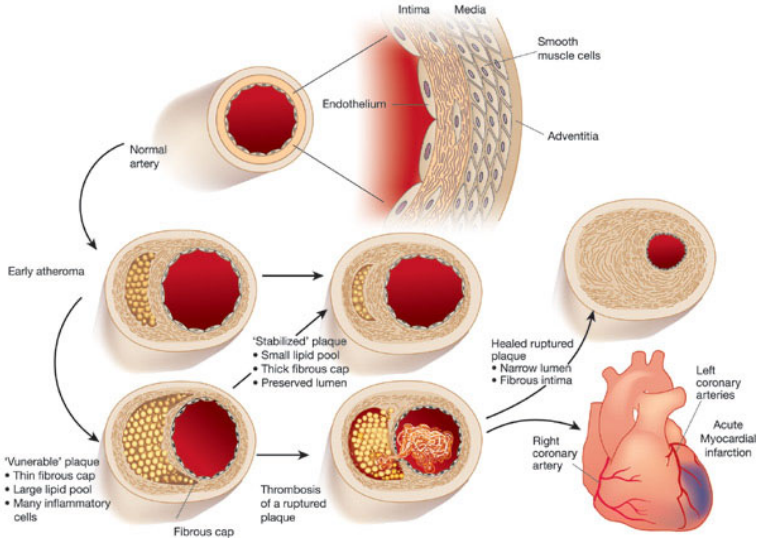

Figure 1.2: The development of atherosclerosis: from a normal artery to a myocardial infarction. During the second decade of life normal arteries start to transform and early atheromas are formed. After this initial stage they can develop into stabilized or vulnerable plaques. The vulnerable plaque can rupture and cause thrombus formation. This can result in an acute myocardial infarction and subsequently in death (adapted from Libby)[28]

Immune cells involved in atherosclerosis

As mentioned above, a significant number of cells and molecules are involved in the immune response during the initiation and the progression of atherosclerosis. To discuss all cell types and molecules lies outside the scope of this thesis and therefore we focused on a few but very important cell types which will be described in separate sections below.

Monocytes/Macrophages

As described above, monocytes and macrophages play an important role in the initiation of atherosclerosis. Next to the vascular smooth muscle cells, macrophages are the most abundant cell types within the lesion. They are part of the innate immune response, normally responsible for the first line defense. The uptake of oxLDL by macrophages via scavenger receptors (CD36, CD68, CXCL16, lectin type oxLDL receptor 1, SR-A and SR-B1) not only leads to activation resulting in inflammatory macrophages but also in the formation of foam cells.[15] Macrophages process the ingested oxLDL and oxLDL derived epitopes may be presented on MHC class II molecules present on the surface of macrophages. This may lead to the activation of antigen specific $CD4^+$ T cells (Figure 1.1). Blocking MHC class II molecules with antibodies resulted in an inhibited oxLDL-induced IFN-γ secretion and T cell proliferation.[29] Therefore, macrophages are considered to be one of the important antigen presenting cells (APCs) in atherosclerosis.

The role of T cells will be discussed in a separate section. Macrophages can also become activated via toll-like receptors (TLRs).[30] These receptors recognize bacterial toxins such as LPS, stress proteins such as heat shock protein 60 (HSP60) and also oxLDL.[31,32] The activated macrophages produce inflammatory cytokines such as TNF-α that is pro-atherogenic. In human atherosclerotic lesions many cells expressing a spectrum of TLRs are found.[33] Intervention in the signaling pathway of TLRs and a lack of TLR4 reduces atherosclerosis in apoE$^{-/-}$ mice.[34,35].

Dendritic cells

The most potent APCs are not macrophages but dendritic cells (DCs). DCs perform multiple important roles in both the innate and the adaptive immune responses.[36] Immature DCs can capture antigens at the site of inflammation and subsequently migrate, triggered by pro-inflammatory signals, to lymphoid organs. During migration they maturate and they subsequently present the captured antigen to naive T cells.[37] Several chemokines such as CCR1, CCR2, CCR5, CCR6, CCR7, CXCR1 and CXCR2 are important regulators of DC-trafficking.[38,39] An important step in the maturation of the DCs is the upregulation of CD40 and subsequently the interaction between CD40, expressed on the DCs and CD40L, mainly expressed on CD4$^+$ T cells. This ligation results in proliferation of T cells and plays a crucial role in immune responses.[40] Upon maturation, DCs also upregulate the expression of several other maturation markers such as CD80 and CD86 and antigen presenting molecules such as MHC class I and II molecules and CD1 molecules. Depending on the local microenvironment of the DCs, they can induce Th1 or Th2 differentiation. In presence of Th1 cytokines such as IL-12, DCs induce a Th1 response.[41] On the other hand, IL-6, IL-13 and OX40-ligand (OX40L) may induce Th2 responses.[42] Chemokines are not only important in trafficking of DCs but also in regulating the maturation of DCs. Maturation of DCs is impaired when CCL3, CCL19 and CCL21 are not expressed.[43,44] Importantly, IL-10 and TGF-β are able to convert Th1-inducing DCs to DCs inducing Th2 or regulatory T cells. The DCs that can induce regulatory T cells are called tolerogenic DCs and produce high levels of IL-10 and TGF-β and low levels of IL-12. These DCs, which are CD11c$^+$ and CD11b$^+$ are present in the Peyer's patches in the intestines and are important in tolerance induction[45] (see below).
In healthy arteries, low numbers of DCs are present within the intima, immediately beneath the endothelium and in the adventitia along the external elastic lamina.[46,47] These subendothelial DCs may be part of the so-called vascular associated lymphoid tissue.[48] The immature DCs scavenge the vessel wall for antigens and within the intima they may ingest atherosclerosis-related antigens.[26,49] During atherosclerosis, the subendothelial DCs increase in number, especially in the shoulder regions of the plaque.[26] Circulating monocytes attracted into the lesion due to chemokines can also differentiate into DCs and additionally, DCs can migrate from the adventitia into the intima. The presence of the DCs in the shoulder regions of the plaque may add to the induction of vulnerable lesions. Within the shoulder region of the lesions, DCs form clusters with activated T cells and NKT cells.[26,50,51] During the maturation

process, the DCs normally migrate due to upregulation of CCR7 and return to secondary lymphoid organs (lymph nodes, spleen).[52] In the lymphoid organs, the maturated DCs present their ingested antigens to T cells. However, due to inhibitory signals generated by PAF and oxLDL, hypercholesterolemia may lead to the activation within the atherosclerotic lesion and to reduced migration of monocyte-derived DC-like cells from the lesion to the secondary lymphoid organs.[53] These DCs stay within the intima and interact with T cells which aggravate the inflammation within the lesion and promote thereby progression of atherosclerotic plaque formation.[54]

Autoantigens in atherosclerosis

As described, both macrophages and DCs are important APCs in atherosclerosis. Both cell types can internalize various substances among which proteins and peptides, (glyco)lipids, etc. which can be processed by the APCs. Epitopes of these antigens can be presented on antigen presenting molecules. In atherosclerosis several antigens of both exogenous and endogenous sources have been identified. Studies indicate that possible antigens derived from exogenous sources play a contributory role in the inflammatory response in atherosclerosis. The most extensively studied pathogens in atherosclerosis are *Helicobacter pylori*,[55] Cytomegalovirus (CMV)[56] and *Chlamydia pneumoniae*[57–59]. Patients with cardiovascular diseases have high antibody titers against these pathogens[60–62] and *Chlamydia pneumoniae*, herpes simplex virus and CMV have been detected within the atherosclerotic plaque.[63] The role of these bacteria and viruses remains however controversial because many contrasting studies exist.
A T cell response to endogenous antigens can be elicited due to molecular mimicry, a cross reaction between the immune response to a pathogenic organism and homologous self-proteins.[64] Heat shock proteins (HSPs) are expressed and secreted by pathogens such as *Chlamydia pneumoniae* and *Helicobacter pylori*. An immune response against microbial HSP65 can cross react with self-HSP60 and thereby induce an autoimmune response. This is due to the fact that HSPs form a family of evolutionary highly conserved proteins.[65] In mammals, HSPs are expressed on endothelial cells and macrophages[66,67] and can be induced by several stress factors such as fluid shear stress,[68] oxidized lipoproteins[69] and cytokines.[67] Under these circumstances, HSPs repair or prevent degradation of denaturated proteins and increase the survival of a cell in response to stress stimuli.[70,71] However, HSP, such as HSP60, are also involved in inflammatory diseases, probably resulting from their increased expression in cells exposed to pro-inflammatory mediators.[72,73] Expression of HSP60 on endothelial, vascular smooth muscle and mononuclear cells is enhanced in human atherosclerotic lesions[74] and patients with cardiovascular diseases have increased titers of HSP60-specific antibodies.[67] These antibodies may contribute to endothelial damage and the inflammatory response in the vessel wall accelerating atherosclerosis.[75] Additionally, HSP60 is expressed by macrophages exposed to oxLDL.[69] Most importantly however, T cell clones with anti-self-HSP60 reactivity have been detected within atherosclerotic lesions.[76] Further on, the induction of tolerance to HSP65 showed to be protective for

atherosclerosis in LDLr$^{-/-}$ mice[77,78] while immunizing mice and rabbits with HSP60/65 accelerated fatty streak formation.[79,80]
Another possibility to develop an autoimmune response is by alteration of self-proteins. The modification of LDL in the vessel wall and in the circulation is thought to contribute to the immune response in atherosclerosis via this mechanism.[81] For example, reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) produced by lipid peroxidation may bind to apolipoprotein B (apoB) resulting in immunogenic oxidative-specific neo-epitopes.[82] The aldehydes can also cross-link amino acids creating many structural variations that may be identified as neo-epitopes. Oxidation of LDL can also result in oxidized phospholipids. The best-known altered self-proteins contributing to the immune response in atherosclerosis are therefore oxLDL and malondialdehyde modified LDL (MDA-LDL). In addition to activation of macrophages and endothelial cells, oxLDL is known to induce T cell activation.[83] OxLDL-specific T cells are detected in the vessel wall and plaques of atherosclerosis patients.[84,85] The presence of antibodies to oxLDL in plasma of animal models for atherosclerosis (LDLr$^{-/-}$ and apoE$^{-/-}$ mice)[86] and in serum of patients with cardiovascular diseases[87] again prove that oxLDL is one of the autoantigens in atherosclerosis. In animal models, the oxLDL-specific antibodies are mainly IgG2a antibodies, indicating Th1 assistance.[86] Anti-oxLDL and anti-MDA-LDL IgG antibodies induced after immunization with oxLDL or MDA-LDL are shown to protect against atherosclerosis due to an enhanced Fc-dependent removal of the antigens by macrophages. This may prevent oxLDL and MDA-LDL from exerting pro-inflammatory and toxic effects in the vascular wall.[17,88–90] In addition, IgM antibodies against oxidized phospholipids in oxLDL also protect against atherosclerosis.[91] In most of these studies and in this thesis, oxLDL is made by copper oxidization or by modification with MDA. The important difference between these forms of oxidization is that copper oxidization results in many different specific epitopes, including MDA and HNE-epitopes and oxidized phospholipids, while MDA-modified LDL contains mainly MDA-epitopes. Immunization of atherosclerosis-prone mice with LDL and modified forms of LDL such as oxLDL or MDA-LDL and with peptide sequences of oxLDL resulted in a reduction of atherosclerosis.[17,88–90,92]
The third protein recognized as a possible autoantigen in atherosclerosis, is β2-glycoprotein I (β2GPI). Normally, this phospholipid-binding protein, which is present on platelets and endothelial cells, acts as an anti-coagulant molecule. However, β2GPI is abundantly expressed within subendothelial regions of human atherosclerotic plaques. Furthermore, CD4^{+} T cells colocalize with β2GPI and this supports the hypothesis that β2GPI initiates a chemoattractant process that results in the attraction of antigen-specific lymphocytes into the atherosclerotic plaque.[93] Additionally, β2GPI colocalizes with oxLDL within the atherosclerotic plaque and these oxLDL/β2GPI complexes may be associated with systemic and chronic inflammation of the vasculature.[94] Antibodies to β2GPI are correlated with the incidence of atherosclerosis in patients and autoantibodies to oxLDL/β2GPI complexes have been associated with arterial thrombosis.[95] A role for β2GPI in atherosclerosis was further indicated by the fact that oral tolerance induction to β2GPI reduced atherosclerosis in LDLr$^{-/-}$

mice[96], while immunization of LDLr$^{-/-}$ mice with human β2GPI accelerated lesion formation.[97]
Most of the above mentioned antigens are based on a peptide structure. These epitopes are presented via MHC class I or II molecules and result in the activation of specific CD8$^+$ or CD4$^+$ T cells, respectively. The activation of these peptide-antigen-specific T cells most likely occurs in the secondary lymphoid organs such as lymph nodes and the spleen. The consequence of T cell activation on atherosclerosis is discussed in detail in the next section.
In addition to the expression of MHC class molecules, the APCs also express another antigen presenting class of molecules, the CD1 molecules. The family of CD1 molecules is related to MHC class I molecules but in stead of presenting protein/peptide antigens they present lipid antigens to T cells.[98] The CD1 family can be subdivided in two main groups. Group 1 molecules, CD1a, CD1b and CD1c, present foreign lipid-antigens to CD1 specific T cells. These molecules are absent in mice. Mice only express molecules from group 2, CD1d, which activates a specific subset of T cells; the natural killer T (NKT) cells, and also CD1d-restricted T cells. Because atherosclerosis is a lipidic disorder, one may imagine that lipid antigens exist in atherosclerosis. It was shown that within the atherosclerotic plaque, CD1d is expressed on DCs that colocalize with T and NKT cells in the shoulder regions of the plaque.[50] In human atherosclerotic plaques, CD1d has been detected incidentally.[99] The role of NKT cell activation by the presentation of (glyco)lipids and the role of CD1d in atherosclerosis will be discussed later.

CD4$^+$ and CD8$^+$ T cells

It was already described in 1985 that T cells are present within human atherosclerotic plaques and that they play a role in the disease process of atherosclerosis.[100] Within human atherosclerotic plaques most of the T cells are CD45RO-expressing effector and/or memory T cells.[101] Almost no naive T cells are found within lesions and the amount of activated T cells increases with the severity of coronary syndrome.[102,103] Most of the T cells are CD4$^+$ T cells[104] and the majority of those are TCRα/β positive, although TCRγ/δ T cells are also present.[80,101] In atherosclerotic lesions of apoE$^{-/-}$ and LDLr$^{-/-}$ mice, the CD4$^+$ T cell is also the predominant T cell subset. A deficiency in CD4$^+$ T cells[105–107] or TCRα/β^+ T cells[108] and thus a deficiency in adaptive immunity leads to reduced atherosclerosis in the atherosclerosis-prone mice. A transfer of CD4$^+$ T cells to immune-deficient scid/scid mice accelerates atherosclerosis, indicating the important role of CD4$^+$ T cells in the disease.[105] Depletion of CD4$^+$ T cells via antibody administration reduced fatty streak formation in C57Bl/6 mice, just like CD4$^{-/-}$ C57Bl/6 mice were protected against fatty streak formation.[109] The activation of the T cells is mainly important in the early progression of atherosclerosis, not in the initiation.[110] CD8$^+$ T cells have also been detected within the human atherosclerotic lesions,[104] but contrasting data on their role in atherosclerosis exist. The absence of CD8$^+$ T cells (apoE$^{-/-}$CD8$^{-/-}$ mice) has no effect on lesion formation,[108] while in another study an acceleration of atherogenesis due to CD8$^+$ T cell activation is observed.[111] Altogether, their

precise role in atherosclerosis remains poorly understood.
The TCRα/β positive CD4$^+$ T cells can be subdivided in several subclasses depending on the cytokine secretion (Figure 1.3). Cytokines are small proteins and peptides that are used as signaling compounds between cells. Many cytokines, both pro-inflammatory and anti-inflammatory, are important regulators of the autoimmune process in atherosclerosis. T helper 1 (Th1) cells, partly responsible for cell-mediated immunity, secrete Th1-cytokines such as IL-1, IL-2, IFN-γ, TNF-α, IL-12 and IL-18, while Th2 cells produce IL-4, IL-5 and IL-13 and may be responsible for antibody production by B cells. Due to high IL-12 and IFN-γ levels during atherosclerosis, most of the CD4$^+$ T cells within the plaque are shown to be of a Th1 cell type and produce TNF-α, IL-2 and IFN-γ. These Th1-cytokines stimulate macrophages and other cells within the lesion to secrete more pro-inflammatory cytokines.[104] IFN-γ enhances also the recruitment of T cells and macrophages to the plaque, increases lipid uptake by the macrophages and activates APCs. Deficiency in IFN-γ or the IFN-γ receptor resulted in attenuated atherosclerosis,[112,113] while injections with IFN-γ accelerates the disease.[114] Within the atherosclerotic plaque, IFN-γ is not only produced by Th1 cells, but also by the later on described NKT cells. Also deficiency in IL-12 and IL-18 in apoE$^{-/-}$ mice leads to an attenuation of atherosclerosis[11,115], whereas treatment with IL-12 and IL-18 accelerated atherosclerosis.[116,117] Additionally, vaccination against IL-12 was successfully used to abrogate the Th1 mediated immune response in atherosclerosis. This effect was due to lowering of especially IFN-γ levels.[118] TNF-α, also produced by macrophages and other cell types, show pro-inflammatory properties and promotes many autoimmune diseases. TNF-α deficiency also results in a reduction of atherosclerosis.[119]

On the opposite side, Th2 cytokines are known for their atheroprotective characteristics, although there are some conflicting data. IL-5 is a promoter of B-1 cell development, the producer of natural antibodies. For example, some of the natural occurring (innate) IgM antibodies cross-react with oxLDL and inhibit cholesterol uptake and foam cell formation.[121] Deficiency in IL-5 leads to an enhanced plaque formation in LDLr$^{-/-}$ mice.[91] IL-4, a so-called Th2 cytokine, is able to inhibit Th1 induced immune responses and therefore protective against a number of Th1-mediated autoimmune diseases. In atherosclerosis, the role of IL-4 is however more complex. IL-4 has athero-promoting characteristics. Recent studies showed a reduced plaque formation in IL-4$^{-/-}$LDLr$^{-/-}$ and IL-4$^{-/-}$apoE$^{-/-}$ mice.[122,123] Van Wanrooij et al. showed that blocking of the OX40 pathway results in a reduced plaque size in LDLr$^{-/-}$ mice due to lower levels of IL-4 and an increased formation of IgM antibodies.[124] IL-4 can cause increased lipid oxidation, enhanced leukocyte adhesion and recruitment and increased lipid uptake and foam cell formation. IL-4 can also cause plaque destabilization by inducing apoptosis of SMCs and MMP-12 production.[125] On the other hand, IL-4 also has some anti-atherogenic properties. Injecting C57Bl/6 mice with IL-4 decreased fatty streak formation.[109] It seems that the effects of IL-4 are stage-dependent, being anti-atherogenic in early atherosclerosis and pro-atherogenic in advanced stages.

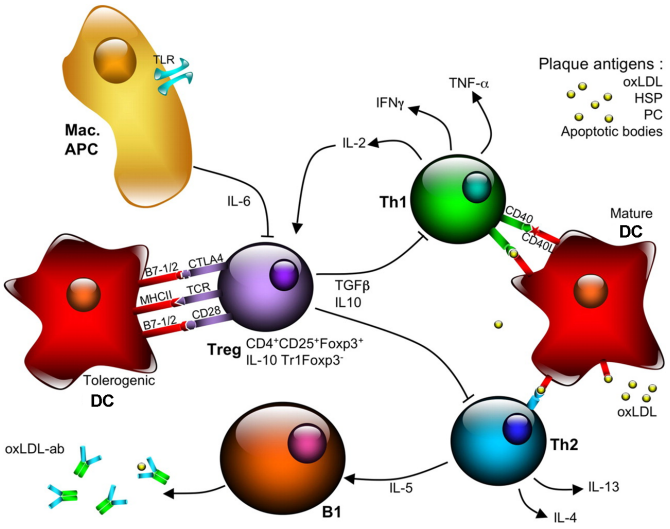

Figure 1.3: An overview of the different CD4$^+$ T cells in atherosclerosis. Upon recognition of a specific antigen presented on MHC molecules by APCs and in presence of co-stimulatory signals, Th1 and Th2 cells secrete specific cytokines. Upon activation Tregs secrete both IL-10 and TGF-β. Both cytokines can inhibit the activation of both Th1 cells producing IFN-γ, IL-2 and TNF-α and Th2 cells producing IL-4, IL-5 and IL-13. (adapted from Tedgui and Mallat) [120]

From the discovery of the role of T cells in atherosclerosis it is thought that the main problem in the immune response is the disbalance between Th1 and Th2 cells. In atherosclerosis, this balance is directed towards Th1. This is supported by the fact that C57Bl/6 mice which are prone to a Th1 type of immune responses develop fatty streaks when they are fed a high cholesterol diet. In contrast, BALB/c mice which are prone to Th2 immune responses are protected against atherosclerosis. [109,126] Many studies described in this section are performed to recover this balance and are done in either normal atherosclerosis-prone C57Bl/6 mice or in transgenic more atherosclerosis-susceptible apoE$^{-/-}$ and LDLr$^{-/-}$ mice.

Regulatory T cells

Recently there was however a change of view on the disturbed Th1/Th2 cytokine balance. IL-10 and TGF-β were found to be very effective in reducing atherosclerosis. Both are anti-inflammatory cytokines that are produced by a distinct subset of T cells; the regulatory T cells (Figure 1.3). IL-10, also produced by macrophages and DCs is produced within the plaque and inhibits oxLDL-induced production of IL-12 by human monocytes [127] and it promotes plaque stability. In addition, endogenous IL-10 [128], systemically and locally administered IL-10 [129] and T cells overexpressing IL-10 [130] are protective against atherosclerosis. Deficiency in IL-10 in C57Bl/6 mice that are fed a atherogenic diet develop increased quantities of fatty streaks [128,131], while IL-10 transgenic C57Bl/6 mice do not develop fatty streaks. Deficiency in IL-10 in apoE$^{-/-}$ mice also results in bigger lesions. [132] TGF-β is the rising star in anti-atherosclerotic

research. TGF-β neutralizing antibodies[133], soluble TGF-β receptors[134] and adenovirus-mediated delivery of TGF-β[135] demonstrates that TGF-β is a potent inhibitor of atherosclerosis. Crossbreeding of apoE$^{-/-}$ mice with mice deficient in TGF-β receptors (TβRII$^{-/-}$) shows a five-fold increase in plaque size.[136] The main functions of TGF-β are lesion stabilization through the induction of the synthesis of collagen and tissue inhibitors of MMPs, dampening atherogenic T cell responses and inhibition of leukocyte recruitment and foam cell formation. Many cells present in the atherosclerotic lesion are capable of producing TGF-β, but the most likely cell type producing this anti-atherogenic molecule is the regulatory T cell.[137,138]
Mallat et al. now hypothesized that in atherosclerosis an imbalance exists between Th1/Th2 effector T cells and the regulatory T cells (Tregs)(Figure 1.4). In the past few years, these so-called Treg cells were identified to be responsible for a unique self-tolerance mechanism and control of autoimmunity. These cells can actively suppress immune activation and maintain immune homeostasis. They are specialized in suppression of both Th1 and Th2 pathogenic immune responses against self or foreign antigens.[139] The majority of Tregs are CD4$^+$ cells and constitutively express CD25 (IL-2Rα). This CD25 is necessary for the development in the thymus, for survival and the function of Tregs. Activation of Tregs via IL-2, produced by non-Treg cells, results in the suppression of pathogenic T cells. The suppression of these T cells leads to the inhibition of IL-2 production and a reduction in the number of Tregs. Thus IL-2 is responsible for the feedback mechanism between pathogenic T cells and Tregs.

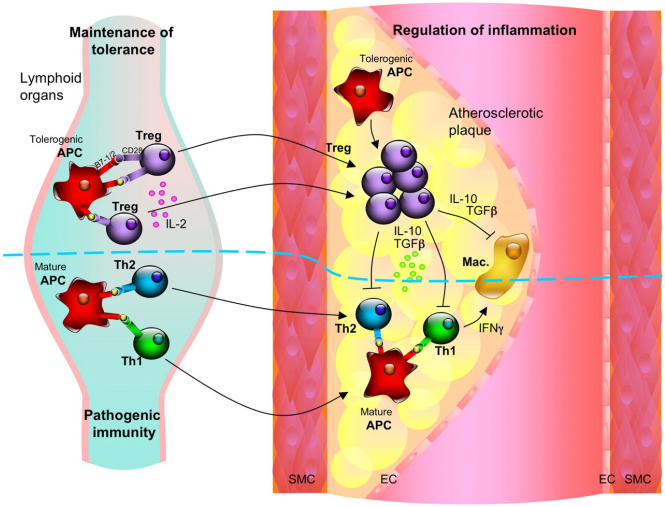

Figure 1.4: The proposed anti-atherogenic properties of Tregs. Tregs may be activated in lymphoid organs by tolerogenic APCs and subsequently migrate to the atherosclerotic lesion just like Th1 and Th2 cells. Upon restimulation in the lesion the Tregs secrete IL-10 and TGF-β. These cytokines inhibit both Th1 and Th2 cells and also macrophages are affected. Altogether this results in a dampened inflammation in the lesion (adapted from Tedgui and Mallat)[120]

Tregs can be subdivided in several types. The common ancestor is located in the

thymus and there they can develop into Foxp3$^+$ or Foxp3$^-$ Tregs. Subsequently, Foxp3$^+$ Tregs, called natural Tregs, migrate out of the thymus into the periphery. The forkhead transcription factor Foxp3 is crucial in the development and function of these natural Tregs.[140] Foxp3 controls several important genes such as CD25, cytotoxic T-lymphocyte antigen-4 (CTLA-4) and glucocorticoid-inducible tumor necrosis factor (GITR).[141] The immunosuppressive function of these Tregs is partly mediated by TGF-β and IL-10 which can have direct effects on other cell types (bystander activation). TGF-β is also expressed on the surface of the natural Tregs instead of being secreted. The most important way of immuno-suppression by natural Tregs is through cell-cell contact via CTLA-4 on the Tregs and B7 on activated APCs and surface bound TGF-β on the Tregs and TβRII on other T cells, or it can be a combination of cell-cell contact and cytokine secretion.[139,142] *In vivo*, activation of Tregs can result in the induction of IL-10 in CD4$^+$CD25$^-$ T cells and TGF-β produced by the Tregs may inhibit pathogenic CD8$^+$ T cells.
The Foxp3$^-$ Tregs also migrate out of the thymus. These cells may be induced in the periphery and are called adaptive Tregs. These can be subdivided again, based on the cytokine they produce most excessively and on the surface markers they express. Tr1 cells (CD4$^+$CD25$^-$), which develop under influence of IL-10, produce mainly IL-10.[143] Th3 cells (CD4$^+$CD25$^+$) are TGF-β producers. The Tr1 cells are CD4$^+$CD25$^-$Foxp3$^-$. Th3 cells express both CD4 and CD25 and may express Foxp3 upon activation. There are contrasting studies about this!
Foxp3-expressing T cells are found in human atherosclerotic plaques[144] and Foxp3 mRNA is expressed in the aorta of apoE$^{-/-}$ mice.[145] The protective action of CD4$^+$CD25$^+$ Tregs is proven by injections with anti-CD25 antibodies in apoE$^{-/-}$ mice.[146] Anti-CD3 Fab antibodies stimulate the activation and proliferation of Tregs and treatment of LDLr$^{-/-}$ mice with these antibodies resulted in a reduction of the development and progression of atherosclerosis.[147] Depletion of several co-stimulatory molecules such as CD28, CD80 and CD86 resulted in a reduction in numbers and function of Tregs and subsequently in higher atherosclerosis susceptibility.[146] Furthermore, a transfer of Tr1 cells specific for ovalbumin elevated IL-10 levels in apoE$^{-/-}$ mice and reduced lesion formation.[148] Recently it was shown that HSP60-specific regulatory T cells can be generated *in vitro* and injection of these cells in apoE$^{-/-}$ mice prevented atherosclerotic lesion development.[149]

Th17 cells

As described, it was assumed for a long time that T cell-mediated tissue damage was caused by a disbalance in Th1 and Th2 cells (Th1/Th2 hypothesis). Later on, Mallat et al. introduced the new view on this balance in atherosclerosis. They postulated that a disbalance between pathogenic T cells and Tregs exist in atherosclerosis (Treg hypothesis). This view on the immunological process in atherosclerosis changed again very recently. Cua et al. revisited the immunopathological basis for diseases such as experimental autoimmune encephalomyelitis (EAE) and collagen induced arthritis (CIA). Traditional blocking of IL-12 did not result in protection against these diseases, but blocking of a specific subunit of IL-23, which shares the subunit p35 with IL-12, resulted in

an attenuation of both diseases.[150] Harrington et al. and Park et al. showed that development of Th17 cells from naive cells is potently inhibited by IFN-γ and IL-4, typical Th1 and Th2 cytokines, respectively, whereas already committed Th17 cells are resistant to suppression by Th1 or Th2 cytokines.[151,152] Further studies defined more functional and phenotypic differences between the so-called Th17 cells and Th1 cells. This new lineage of T cells, Th17 cells, are now credited for causing tissue damage.[153] Th17 cells are driven by IL-23 and TGF-β in presence of IL-6 and they mainly produce the pro-inflammatory cytokine IL-17. IL-17 deficient mice show attenuated disease progression of EAE[154] and suppressed collagen-induced arthritis.[155–157] Van Es et al. showed that vaccination against IL-17 protects against atherosclerosis.[158] The new view on the balance of T cells is now called the Th17 hypothesis with Th17 cells on one side and Tregs on the other. IL-6 and TGF-β seems to be key-players in this balance since TGF-β plus IL-6 initiates Th17 cells, while TGF-β without IL-6 initiates Tregs (Figure 1.5).

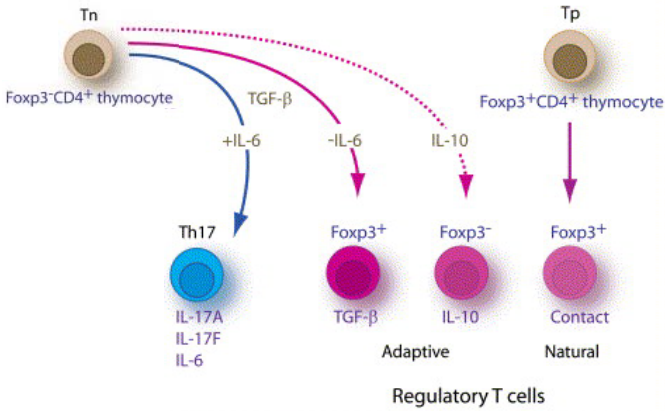

Figure 1.5: The new view on the balance between T cells in atherosclerosis. Th17 cells and Tregs may develop from a common Foxp3$^-$CD4$^+$ ancestor. In presence of TGF-β and IL-6 the ancestor differentiates into th17 cells, while without IL-6 they differentiate into Tregs. Th17 cells produce pro-atherogenic IL-17 and IL-6, while Tregs may produce NT-atherogenic TGF-β and IL-10.(adapted and modified from Weaver et al.[159])

NKT cells

Besides recognition of peptide antigens by T cells and Tregs, lipid antigens can be recognized by another specialized subset of T cells, the NKT cells. NKT cells are characterized by the expression of NK cell receptors such as CD161 (NK1.1 in mice) and an invariant TCR composed of Vα14 and Jα18 (previously known as Jα281) subunits paired with a restricted set of Vβ chains. Most of the NKT cells are CD4$^+$ or CD4$^-$CD8$^-$.[160–162] These classical NKT cells can recognize (glyco)lipid antigens presented by CD1d expressed on APCs. NKT cells are present in both humans and mice and in mice they are found most frequently within the liver (30-50% of the lymphocyte population) and bone-marrow (20-30%). They represent a smaller proportion of lymphocytes in the spleen (3%), lymph nodes (0.3%), blood (0.4%) and lung (7%). Within the liver they reside in

the sinusoids where they screen for possible ligands. Several subsets of NKT cells are known at the moment. The CD1d dependent NKT cells can be subdivided in NK1.1$^+$ and NK1.1$^-$ cells and there are also CD1d dependent NKT cells without expression of Vα14-Jα18 and CD1d independent but NK1.1$^+$ NKT cells. The most common NKT cell is the invariant NKT cell, which is CD1d dependent and expresses NK1.1. Upon recognition of a glycolipid antigen, invariant NKT cells rapidly produce large amounts of both Th1 (IFN-γ, IL-12 and TNF-α) and Th2 (IL-4, IL-5, IL-10 and IL-13) cytokines and this makes them unique among lymphocytes[163] (Figure 1.6). The question remains how this mix of cytokines can lead to a regulated immune response. One possible explanation could be the difference in the affinity of the ligand for CD1d, which can result in different signaling inside the NKT cell (see below). An important feature of the released cytokines from NKT cells is the bystander activation of adjacent NK cells, B cells and dendritic cells[164] as well as activation of normal CD4$^+$ and CD8$^+$ T lymphocytes.[160] Recent studies have shown that NKT cells can be activated by *in vivo* administration of marine sponge-derived α-galactosylceramide (α-GalCer) also known as KRN7000. Upon recognition of α-GalCer, mature NKT cells induce an early burst of IL-4 followed by a more prolonged burst of IFN-γ.[165] However, repeated injections of α-GalCer have been shown to induce an adaptive immune response which is polarized towards production of Th2 cytokines (IL-4, IL-10). This results in the protection against autoimmune diabetes,[166] experimental autoimmune encephalomyelitis[167] and colitis[168] in mice (Table 1.1). Interestingly some variants of α-GalCer exist that have decreased Th1 compared to Th2 cytokine induction. OCH, which has a truncated sphingosine chain, is one of these α-GalCer analogs. *In vitro* administration of OCH results in a higher ratio of IL-4 to IFN-γ secretion by NKT cells[165,169] and *in vivo* it results in the prevention of experimental autoimmune encephalomyelitis,[165] diabetes[170] and collagen-induced arthritis.[171] Also the production of IL-10 by OCH-stimulated NKT cells is beneficial in Th1-mediated autoimmune diseases[170] (Table 1.1). It has been suggested that the differences in cytokine profiles may be due to the length of the sphingosine chain. Due to the shorter lipid chain of OCH, it has a lower affinity for CD1d. This results in a shorter TCR ligation which results in a poor transcription of the NF-κB family member transcription factor c-Rel. c-Rel is identified as essential for IFN-γ production by NKT cells. It was observed that c-Rel is transcribed in α-GalCer-stimulated, but not in OCH-stimulated NKT cells so this may be responsible for the reduced IFN-γ gene transcription.[172] An alternative hypothesis is that the different ligands reach different cell types upon injection. Th1 responses may result from the uptake of the ligand by for example IL-12 secreting cells such as certain DC-subsets, whereas Th2 responses may result from the uptake by non-IL-12 producing cells such as B cells or other DC-subsets.[173,174]

The aforementioned antigens are all synthetically made. Recently some microbial ligands were found. Glycosphingolipids in the cell wall of *Sphingomonas* strongly activate NKT cells and NKT cells clear the infection of these bacteria.[176–178] In addition, more recently also a self-ligand for NKT cells was found. The lysosomal glycosphingolipid iGb3 may activate mouse Vα14 and human Vα24 NKT cells. This iGb3 seems to be important during the natural development of NKT cells.

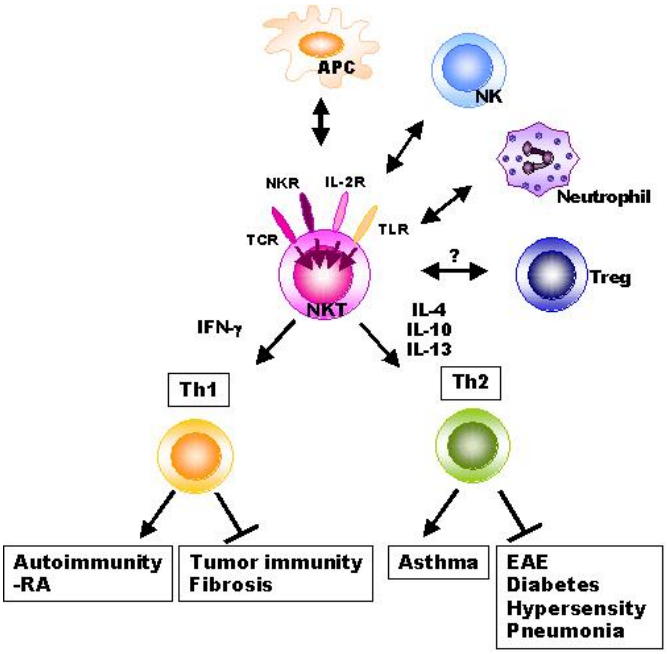

Figure 1.6: Production of both Th1 and Th2 cytokines by activated NKT cells. Upon recognition of ligands such as α-GalCer and OCH by CD1d on APCs, NKT cells may produce a large diversity on both Th1 (IFN-γ) and Th2 (IL-4, IL-10 and IL-13) cytokines. These cytokines may induce bystander activation of Th1 and Th2 cells and this results in the initiation or prevention of different inflammatory diseases.

It is present within the thymus and is necessary during selection and is CD1d dependent.[179,180] In addition to the microbial and lysosomal ligands, recently some plant- and bacteria-derived glycolipids were found to be possible ligands. Especially the phosphatidyl choline (PC) and phosphatidyl ethanolamin (PE) parts of these glycolipids are shown to activate NKT cells.[181,182] Whether all these "natural" ligands affect the Th1/Th2 cytokine secretion of NKT cells and whether they can be used as treatment for diseases needs further investigation.[183]
Because of the activation of NKT cells by (glyco)lipids and the fact that (glyco)lipids are a major part of the problems in atherosclerosis, NKT cells may play a role in atherosclerosis. Several groups are active in this field and both α-GalCer and OCH were used to study the effects of NKT cell activation on atherosclerosis. The first publication on a possible role for NKT cells in atherosclerosis showed that apoE$^{-/-}$ mice crossed with CD1d$^{-/-}$ mice exhibit a 25% reduction in lesion size. α-GalCer treatment resulted in a burst of cytokines and induced a 50% increase in lesion size.[184] These data are confirmed by the study of Major et al.[185] In both studies IL-4 and IFN-γ production was increased after treatment. LDLr$^{-/-}$ mice crossed with CD1d$^{-/-}$ mice also have smaller lesions compared with LDLr$^{-/-}$ mice.[186,187] It is however mentioned that this effect was only present during the initial stages of atherosclerosis. There was an effect after 4 weeks of diet, while the effect of CD1d deficiency was lost after 8 and 12 weeks of diet.[187] Nakai et al. repeatedly injected apoE$^{-/-}$ mice with both α-GalCer

Disease	Mouse strain	Model	Treatment protocol	Treatment Outcome	Proposed mechanism
T1D	NOD	spontaneous	α-GalCer,multiple i.p.	protection	Th2 deviation, MDC recruitment
	NOD	spontaneous	α-GalCer + IL-7, multiple i.p.	superior protection	Th2 deviation
	NOD	islet transplant	α-GalCer, multiple i.p.	protection	Th2 deviation
	NOD	CY	α-GalCer, multiple i.p.	protection	Th2 deviation
EAE	B6	MOG(35-55)	α-GalCer, multiple s.c/i.p.	protection	Th2 deviation
	B6	MOG(35-55)	α-GalCer, multiple s.c	protection	NKT cell IFN-γ
	B6	MOG(35-55)	α-GalCer, multiple s.c/i.p.	slight exacerbation	competition for CD1D
	B6	MOG(35-55)	α-GalCer, single i.p.	no effect or protection	Th2 deviation
	B6	MOG(35-55)	APC + α-GalCer + B7.2 blockade, single i.p.	protection	Th2 deviation
	B6	MOG(35-55)	APC + α-GalCer + CD40 activation, single i.p.	exacerbation	Th1 deviation
	B6	MOG(35-55)	OCH, single i.p. or p.o.	protection	Th2 deviation
	PL/J	MBP	α-GalCer, multiple s.c/i.p.	protection	Th2 deviation
	B10.PL	MBP(AC1-9)	α-GalCer, co-immun., single i.p.	exacerbation	Th1 deviation
	B10.P	MBP(AC1-9)	α-GalCer, pre-immun., i.p.	protection	Th2 deviation
	SJL/J	MBP	α-GalCer, multiple s.c/i.p.	slightly delay, increased mortality	Th1 bias of NKT cells
RA	B6, SJL/J	collagen	α-GalCer, multiple i.p.	weak or no protection	Th2 deviation
	B6, SJL/J	collagen	OCH, multiple i.p.	protection	Th2 deviation
SLE	NZB/W F1	spontaneous	α-GalCer, multiple i.p. at adult age	exacerbation	Th1 deviation
	MRL-lpr/lpr	spontaneous	α-GalCer, multiple i.p.	decreased dermatitis, no effect on lupus nephritis	regulatory cytokines
IBD	B6	DSS	α-GalCer, multiple i.p.	weak protection	regulatory cytokines
ATH	B6.ApoE$^{-/-}$	spontaneous	α-GalCer, multiple i.p.	exacerbation	NKT cell recruitment and activation in lesion

Table 1.1: The effect of α-GalCer treatment on different inflammatory diseases. In most inflammatory diseases, multiple injections of α-GalCer or OCH resulted in a protection against the disease. In studies that observed an exacerbation of the disease the ligand was injected only once or was injected at adult age. The only exception in this table is atherosclerosis in which multiple injections of α-GalCer accelerated the disease (adapted from van Kaer) [175]

and OCH during the early phase of atherosclerosis. Both ligands caused an increase in plaque size which was dedicated to an increase in the production of pro-atherogenic cytokines (IL-4 and IFN-γ).[186] The effect of α-GalCer on the development of atherosclerosis and the produced pro-atherogenic cytokines by NKT cells suggests that CD1d-dependent NKT cells may play a specific role in atherogenesis. Furthermore, the results with CD1d$^{-/-}$ mice suggest that the absence of CD1d-reactive NKT cells attenuates atherosclerotic lesion formation in mice during early fatty streak formation.
In addition, NKT cells are found in the aortic arch of LDLr$^{-/-}$ and apoE$^{-/-}$ mice fed a Western-type diet.[184–186,188] In advanced human lesions NKT cells have been identified in the regions bordering the shoulder of the lipid core and the fibrous cap,[189] especially there where DCs are present.[50] In these regions, NKT cells represent 2% of the total lymphocyte population.[50,189]

Immunotherapies as treatment for atherosclerosis

Currently, many research groups around the whole world are struggling to find the best treatment for atherosclerosis. From multiple sides the disease process is "attacked". Lipid lowering therapies, promotion of physical activity, blood-pressure lowering methods and many other techniques are still not sufficient enough to win this battle. With statins and anticoagulants the development of atherosclerosis and its consequences can be inhibited, but still the disease is manifest.[190] Since the knowledge that atherosclerosis has an important inflammatory component, major research effort has been done to interrupt the inflammatory response and thereby reduce the severity of the inflammation and consequently the severity of atherosclerosis.[191] Many approaches are focused on the role of certain cytokines or cell types. Depletion or overexpression of athero-promoting cytokines and athero-protective cytokines, respectively, is shown to be successful in atherosclerosis-prone LDLr$^{-/-}$ and apoE$^{-/-}$ mice (described above). Another upcoming technique to block key players of atherosclerosis is via active vaccination. Using a novel vaccination technique[192], antibodies to IL-12 can be induced which specifically block the function of IL-12 and protect against atherosclerosis in LDLr$^{-/-}$ mice.[118] This vaccination strategy was based on a protein vaccine. Another protein vaccination against cholesteryl ester transfer protein (CETP) altered the lipoprotein profile in cholesterol-fed rabbits and reduced the lesion formation.[193] Hauer et al. also showed that DNA vaccination against the vascular endothelial growth factor receptor 2 (VEGFR2) blocked the initiation and progression of atherosclerosis,[194] whereas van Es et al. showed that DNA-vaccination against IL-17 blocked the initiation of atherosclerosis.[158]
Another important way to interrupt the disease process is by reducing the antigen-specific immune responses in atherosclerosis. This can be done by removing the antigens which is hard to achieve in humans since many antigens are self-proteins. Two other ways are immunization and tolerance induction to specific atherosclerotic antigens. Immunization with oxLDL protects against atherosclerosis in hypercholesterolemic rabbits,[195] while immunization with MDA-LDL and apoB100 peptide sequences reduces atherosclerosis in LDLr$^{-/-}$ and apoE$^{-/-}$ mice, respectively.[88,92]
The different approaches used in this thesis are described below. First tolerance induction to auto-antigens of atherosclerosis is mentioned. This was already successful in several other publications in which oral and nasal tolerance to HSP65 and β2GPI protected against lesion formation in LDLr$^{-/-}$ mice[77,78,96], while induction of neonatal tolerance to oxLDL reduced atherosclerosis in apoE$^{-/-}$ mice.[196] Secondly, a new vaccination approach is introduced to reduce atherosclerosis. This vaccination technique uses DCs as transfer units of specific antigens and ligands. The principles of DC vaccination will be clarified in the second part. At last different strategies to activate NKT cells are mentioned.

Tolerance induction

It is known that Tregs can be induced via mucosal tolerance induction. The classical definition of mucosal tolerance is a specific suppression of cellular

and/or humoral immune responses to an antigen by administration of the antigen via a mucosal surface. It is a form of peripheral tolerance that evolved to treat external agents that gain access to the body via a natural route. It is of unique immunologic importance, because it is a continuous natural immunologic event driven by exogenous antigens. Many processes, both Th1 and Th2-driven, can be suppressed or induced by mucosal tolerance induction.
To induce mucosal tolerance, the antigen has to be administered to a mucosal surface. This can be achieved in two ways; nasal or oral administration. The main pathways in both tolerance mechanisms are the same. It is shown that oral tolerance induction mainly induced Th3 cells[197,198], while nasal tolerance induction mainly resulted in the activation of Tr1 cells. In recent studies it is shown that oral tolerance induction also results in an increase in $CD4^+CD25^+$ cells expressing either CTLA-4 and/or FoxP3.[199,200] In this thesis the method of oral tolerance induction is used in two chapters and therefore this section of the introduction is focused on this method of tolerance induction.
The effects of oral tolerance induction are mainly determined by the dose of the antigen. Low dose feeding results in Treg activation in the gut, while high doses result in deletion/anergy of the antigen specific T cells.[201,202] The antigen-specific Tregs are activated in the gut due to presentation of the fed antigens by tolerogenic DCs.[203] As mentioned earlier, the Tregs mainly producing IL-10 and TGF-β, may migrate to lymphoid organs and target organs to suppress the disease both in an antigen-specific and an antigen-non-specific way.[204] The induced Tregs are specific for the fed antigen and can recognize the antigen in the lymphoid organs or target organs and secrete their cytokines locally. Additionally they can suppress the disease by releasing antigen-non-specific cytokines, which is called bystander suppression.
The method of tolerance induction to treat atherosclerosis is used in several studies. Harats et al. used HSP65 and feeding of certain doses of this antigen induced a specific immune suppression and a reduction in lesion size in $LDLr^{-/-}$ mice which were immunized with *Mycobacterium tuberculosis* or fed an atherogenic diet to induce atherosclerosis. The mechanism underlying this effect was however not clearly defined. Low doses of HSP65 reduced plaque size but without inducing a specific immune suppression.[77] Maron et al. showed a reduction in atherosclerosis after nasal and oral administration of HSP65, but only nasal administration led to a change in T cell phenotype.[78] In a study by George et al, β2GPI was used as atherosclerosis-specific antigen. Oral feeding of this antigen also resulted in a reduction of plaque size. They also show a lowered lymph node cell reactivity to β2GPI and an upregulation in anti-atherogenic cytokines but again the underlying method remains to be clarified.[96]

DC vaccination

DCs are more and more used in vaccination strategies to treat not only cancer but also autoimmune responses in animal models. DCs can be isolated and cultured from different sources such as bone marrow, blood and lymphoid organs. The immature DCs can be loaded with different compounds *ex vivo*. Proteins, peptides and lipids can be taken up and presented via antigen presenting

molecules. DCs can also be loaded with DNA or mRNA, viral vectors, tumor cells and tumor cell lysates. Transferring peptide- or lipid- loaded DCs can result in the induction of a number of responses. In one study, DCs were pulsed with bovine collagen type II and injection of the DCs in mice protected against collagen-induced arthritis.[205] The observed effect was dedicated to a decrease in the collagen-specific Th1-associated IgG2a response. Loading immature DCs with a peptide of glutamic acid decarboxylase and injection of these DCs in non-obese diabetic mice protected against type I diabetes.[206] Furthermore and of importance for this thesis, lipids can be loaded on DCs. This may result in an activation of lipid-specific NKT cells and a prolonged production of IFN-γ by these cells. These DCs were protective against several forms of cancer.[207,208]

α-GalCer and OCH administration

As described above, α-GalCer and OCH are injected to activate NKT cells. In most studies on autoimmune diseases, both ligands are administered intravenously (i.v.), intraperitoneally (i.p.) or a combination of both (Table 1.1). One single injection of α-Galcer mostly results in the activation of NKT cells producing more Th1 than Th2 cytokines. This results in a exacerbation of different diseases in mouse models. Multiple injections however switch the NKT cell activation towards a Th2 phenotype resulting in protection against many autoimmune diseases except atherosclerosis (Table 1.1).
The ligands for NKT cells can also be administered via the above described vaccination technique. Two studies showed that NKT cells can be activated by injecting α-GalCer loaded on mature dendritic cells. This resulted in a sustained expansion of NKT cells and a prolonged production of IFN-γ.[207,208] Both ways of administering NKT cell ligands may be useful to modify the immune response in atherosclerosis.

Outline of this thesis

In this thesis several therapeutic strategies are used to treat atherosclerosis. All these strategies are focused on interruption of the harmful Th1 immune response in atherosclerosis. In all strategies there is a central role for the DCs. First of all DCs were used as a vaccination unit to induce an atheroprotective antibody response and to induce a protective NKT cell activation. Additionally, α-GalCer was used to treat atherosclerosis and since DCs express CD1d they will be very important in this process. Two studies are focused on the induction of oral tolerance to atherosclerosis-specific antigens. Tolerogenic DCs present in the Peyer's patches in the gut are important regulators of the activation of Tregs after oral feeding of the antigens. In the last study, the faith of NKT cells after Western type diet feeding is described. DCs which engulf lipids can be responsible for the activation of NKT cells in atherosclerosis. Overall, the focus of this thesis was to develop novel experimental therapeutic strategies to treat atherosclerosis and to increase insight in the role of Tregs and NKT cells in atherosclerosis.
Chapter 2 describes a study in which oral tolerance induction to oxLDL leads to an increase in oxLDL-specific Tregs in several organs and leads to an amelioration

of early atherosclerotic (30-71%) and advanced atherosclerotic lesions (45%). Furthermore, an increase in Tregs within the atherosclerotic lesion and an increased oxLDL-specific TGF-β production was observed.
Chapter 3 also describes a study in which oral tolerance induction was used to inhibit atherosclerosis. In this study it is shown that tolerance induction to HSP60 or an HSP60 peptide (253-268) reduces atherosclerosis with 81%. After treatment with HSP60, an increase in Foxp3$^+$ Tregs was observed in several organs and the atherosclerotic lesion. In addition, treatment with HSP60 increased TGF-β and IL-10 production by HSP60 specific cells, while splenocytes from HSP60-treated mice show lower proliferation in response to HSP60.
Chapter 4 describes a study in which mice were treated with oxLDL-pulsed DCs. Atherosclerosis was dramatically reduced which may be due to an increased plaque stability, lowered plasma cholesterol levels and increased titers of anti-oxLDL IgG levels in serum of mice treated with oxLDL-pulsed DCs. This anti-oxLDL IgG participates in immune-complex formation and reduces foam cell development.
Chapter 5 describes a study in which the athero-protective effects of α-GalCer were investigated. In contrast with other studies, a reduction in atherosclerosis was found after multiple injections with α-GalCer in high fat diet fed LDLr$^{-/-}$ mice. This effect was not observed in apoE$^{-/-}$ mice. Splenocytes of LDLr$^{-/-}$ mice were more responsive to α-GalCer and Western-type diet feeding induced a more abundant increase in NKT cells in LDLr$^{-/-}$ mice when compared to apoE$^{-/-}$ mice.
Chapter 6 describes another DC-vaccination study in which mice were treated with OCH-pulsed DCs. OCH-pulsed DCs induce an activation of NKT cells, which are increased in the liver and blood. Additionally we observed a Th2 profile of cytokines in the spleen and a lowering of total plasma cholesterol after treatment with OCH-pulsed DCs.
Chapter 7 describes the search for natural ligands of NKT cells. Western-type diet feeding in LDLr$^{-/-}$ mice resulted in an increased number of NKT cells in the liver and spleen. Proliferation studies with LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice showed that oxLDL or at least a part of it can activate NKT cells. However, a similar lesion size in LDLr$^{-/-}$Jα281$^{-/-}$ and LDLr$^{-/-}$ mice fed a Western-type diet for 12 weeks was observed.
Chapter 8 discusses the findings of this thesis and future perspectives of these studies. Also the therapeutic implications will be evaluated.

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Chapter 2

Induction of oral tolerance to oxidized LDL ameliorates atherosclerosis

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Abstract
Oxidation of low-density lipoprotein (LDL) and the subsequent processing of oxidized LDL (oxLDL) by macrophages results in activation of specific T cells, which contributes to the development of atherosclerosis. Oral tolerance induction and the subsequent activation of regulatory T cells may be an adequate therapy for the treatment of atherosclerosis. Tolerance to oxLDL and malondialdehyde-treated LDL (MDA-LDL) was induced in $LDLr^{-/-}$ mice fed a Western-type diet by oral administration of oxLDL or MDA-LDL before the induction of atherogenesis. Oral tolerance to oxLDL resulted in a significant attenuation of the initiation (30-71%; $P<0.05$) and progression (45%; $P<0.05$) of atherogenesis. Tolerance to oxLDL induced a significant increase in $CD4^+CD25^+Foxp3^+$ cells in spleen and mesenteric lymph nodes, and these cells specifically responded to oxLDL with increased transforming growth factor-β production. Tolerance to oxLDL also increased the mRNA expression of Foxp3, CTLA-4 and CD25 in the plaque. In contrast, tolerance to MDA-LDL did not affect atherogenesis. In conclusion, oxLDL-specific T cells, present in $LDLr^{-/-}$ mice and important contributors in the immune response leading to the atherosclerotic plaque, can be counteracted by oxLDL-specific $CD4^+CD25^+Foxp3^+$ regulatory T cells activated via oral tolerance induction to oxLDL. We conclude that the induction of oral tolerance to oxLDL may be a promising strategy to modulate the immune response during atherogenesis and may be a new way to treat atherosclerosis.

Introduction

The uptake of oxidized low-density lipoprotein (oxLDL) in the vessel wall by antigen presenting cells (APCs), such as macrophages and dendritic cells, is one of the hallmarks of the T helper 1 (Th1)-mediated immune response in atherosclerosis. OxLDL ingested by macrophages is processed, and oxLDL-derived epitopes will be presented on the cell surface via major histocompatibility complex (MHC) class I and II molecules. Via the T cell receptor, oxLDL-specific $CD8^+$ and $CD4^+$ T cells bind to MHC class I or II antigen complex, respectively, and this results in the activation and proliferation of oxLDL-specific T cells. The putative role of this oxLDL-specific response in atherosclerosis is established by the presence of oxLDL-specific T cells in the vessel wall and atherosclerotic plaques.[1,2] These T cells are especially Th1 cells producing Th1 cytokines such as interferon- IFN-γ, interleukin (IL)-12, IL-2 and tumor necrosis factor (TNF)-α.[3–5] In addition, immunoglobulin G2a (IgG2a) antibodies against oxLDL, indicating Th1 assistance, predominate in plasma of low-density lipoprotein receptor $(LDLr)^{-/-}$ and apolipoprotein E $(apoE)^{-/-}$ mice during early stages of atherosclerosis.[6] The presence of antibodies to oxLDL in serum of patients with cardiovascular diseases also proves that oxLDL is one of the (auto)antigens in atherosclerosis.[7]

Oral tolerance induction to autoantigens is one of the possible treatments for Th1-mediated autoimmune diseases such as multiple sclerosis,[8,9] rheumatoid arthritis[10,11] and type I diabetes.[12,13] Antigens administered orally enter the gut associated lymphoid tissue (GALT),[14,15] which, as primary function, protects the host from ingested pathogens and proteins.[16,17] Currently, 2 primary effector mechanisms of oral tolerance induction are known. Feeding high doses of an antigen result in anergy or deletion of antigen-specific T cells,[18,19] whereas low doses of an antigen result in the induction of antigen-specific regulatory T cells.[20] These regulatory T cells can be divided into three groups: Th3 cells and Tr1 cells, mediating suppression via secretion of TGF-β and IL-10, respectively, and $CD4^+CD25^+$ regulatory T cells characterized by the expression of the transcription factor forkhead box P3 (Foxp3). The regulatory function of the $CD4^+CD25^+Foxp3^+$ cell is mediated by cell contact and surface-bound TGF-β and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4).[21] After activation in the GALT, the regulatory T cells migrate to the site of inflammation and on reencountering the fed antigen they display their specific suppressive effect, resulting in an attenuated Th1 mediated immune response specific for the fed antigen. Initial studies show that oral tolerance induction to β2-glycoprotein I[22] and HSP65[23,24] results in the suppression of early atherosclerosis and demonstrate that oral tolerance induction could be a successful treatment for atherosclerosis.

In the present study we demonstrate that induction of oral tolerance to oxLDL attenuates both the initiation and the progression of atherosclerosis, whereas malondialdehyde-treated LDL (MDA-LDL) was unable to mediate this effect. The effect of oxLDL tolerance may be explained by a significant increase in $CD4^+CD25^+Foxp3^+$ regulatory T cells in the mesenteric lymph nodes and

spleen, an increased production of TGF-β by these cells and a significant upregulated expression of Foxp3 and CD25 in the atherosclerotic lesions.

Methods

Animals

All animal work was approved by Leiden University and was in compliance with the Dutch government guidelines. LDLr$^{-/-}$ mice were from the Jackson Laboratory, Bar Harbor, Me. They were kept under standard laboratory conditions and administered food and water *ad libitum*.

Antigens and adjuvant

Dimethyl dioctadecyl ammonium bromide (DDA) was from Sigma Diagnostics, St. Louis, MO. LDL was isolated from serum of a healthy volunteer.[25] Isolated LDL was oxidized by 10 μM CuSO4 at 37°C for 20 hrs[26] and MDA-LDL was made by addition of 0.5 M MDA to 10 mg of LDL for 3 hours at 37°C.

Immunizations

LDLr$^{-/-}$ mice were immunized with 100 μg of oxLDL or MDA-LDL together with 100 μg of DDA via one i.p. injection. After 14 days spleens were used in proliferation assays.

Spleen cell proliferation assay

Spleen cells were collected by squeezing the spleen through a 70 μm cell strainer, erythrocytes were removed by an erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Splenocytes were cultured at $2{\cdot}10^5$ cells/well in 96-wells plates in RPMI 1640 (2 mM L-Glutamine, 10% FCS) for 24-48 hours. Concanavalin A (Con A; Sigma-Diagnostics, MO) was used as a positive control. Cultures were pulsed for the final 16 hours with [6-^{3}H]-thymidine (1 μCi/well, Amersham Biosciences, The Netherlands). Subsequently, cells were washed, lysed and [^{3}H]-thymidine incorporation was measured. Responses are expressed as stimulation index (SI): ratio of mean counts per minute of triplicate cultures with antigen to triplicate cultures without antigen.

Flow cytometric analysis

Splenocytes were incubated with oxLDL (1-10 μg/ml) in the presence of anti-CD3 (5 μg/ml) and anti-CD28 (5 μg/ml). 48 hours later cells were harvested, incubated with 1% normal mouse serum and stained with PerCP-conjugated anti-CD3 and FITC-conjugated anti-F4/80 (0.5 μg Ab/200,000 cells). After washing, cells were analyzed by flow cytometry on a FACSCalibur. To detect $CD4^+CD25^+Foxp3^+$ T cells, a three color flow cytometry was performed. Mononuclear cells were isolated from spleen, mesenteric lymph nodes, Peyer's patches, and blood using Lympholyte (Cedarlane, Canada). Cells were stained

with FITC-conjugated anti-CD4 (0.125 μg/sample) and APC-conjugated anti-CD25 (0.06 μg/sample) mAb, cells were washed, fixed and permeabilized. Subsequently cells were stained with PE-conjugated anti-Foxp3 (0.2 μg/sample). Cells were washed and analyzed by FACS. Data were analyzed with CELLQuest software (BD Biosciences, The Netherlands) and antibodies were from eBioscience, Belgium.

Induction of atherosclerosis

Atherosclerosis was induced in LDLr$^{-/-}$ mice by feeding a Western-type diet (0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK)) two weeks prior to placement of perivascular collars.[27] Total cholesterol levels were quantified using an enzymatic procedure (Roche Diagnostics, Germany) using Precipath as an internal standard.

Oral tolerance induction during atherosclerosis

To induce tolerance LDLr$^{-/-}$ mice were fasted for 16 hrs. 2 mg of soybean trypsin inhibitor (STI, Sigma, MO) was administered orally to prevent antigen degradation and 10 minutes thereafter mice orally received PBS, 30 μg of Super Oxide Dismutase, (SOD, Sigma-Diagnostics, MO), 30 μg of oxLDL or MDA-LDL. Injections were repeated 3 times to a total of 4 injections in 8 days. After administering the antigens mice were kept on Western-type diet. This was performed either in the first week of diet or after 10 weeks of diet.

Plaque analysis

Six weeks after collar placement the mice were anaesthetized with ketamine-hypnorm and perfused with FormalFixx. Common carotid arteries and both carotid bifurcations were removed for analysis.[27] Arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and 5 μm sections were made on a Leica Cryostat proximal to the collar. Cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). For analysis of the aortic root 10 μm thick sections were made of the aortic root containing the aortic valves. Sections were stained with Oil-Red-O and hematoxylin. Plaque sizes were measured using a Leica DM-RE microscope and LeicaQwin software. Sections were also stained immunohistochemically using antibodies against a macrophage-specific antigen (MOMA-2, Research Diagnostics Inc) and α-smooth muscle cell actin (monoclonal mouse IgG2a, Sigma) exactly as described.[28]

Cytokine assays

Mesenteric lymph node cells were cultured at $2{\cdot}10^6$ cells/ml with or without 5 μg/ml oxLDL. IL-10, IFN-gamma (eBioscience, Belgium) and TGF-β (Bender MedSystems, Austria) concentrations were determined by ELISA in the supernatants.

Real-time PCR assays

Carotid arteries from control and oxLDL-treated mice were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems, CA) using SYBR green technology. The following primer pairs were used: 5′-GGAGCCGCAAGCTAAAAGC-3′ and 5′-TGCCTTCGTGCCCACTGT-3′ for Foxp3; 5′-CTTATATTGCAAATGTGGCACAATC-3′ and 5′-ATCAATCATCAGTGGGACAATCTG-3′ for CD25; 5′-CGAGGTCCTGCACCAACTG-3′ and 5′-TCCATCACCATCGGTTTATGC-3′ for CTLA4. Acidic ribosomal phosphoprotein PO (36B4) was used as the endogenous reference gene and detected using the primers 5′-GGACCCGAGAAGACCTCCTT-3′ and 5′-GCACATCACTCAGAATTTCAATGG-3′.

Detection of anti-oxLDL antibodies

OxLDL (5 μg/ml) dissolved in a $NaHCO_3/Na_2CO_3$ buffer (pH 9.0) was coated. Measurement of IgG1 and IgG2a levels in serum was performed using an ELISA Ig detection kit (Zymed Laboratories, CA) conform the manufacturer's protocol and appropriate controls were performed.

Statistical analysis

All data are expressed as mean$\pm$SEM. The two-tailed student's t-test was used to compare proliferative responses to antigens, FACS data, differences in cytokine production, and atherosclerotic parameters between the different groups.

Results

T cells specific for oxLDL and MDA-LDL in LDLr$^{-/-}$ mice

The presence of T cells specific for oxLDL and MDA-LDL epitopes in LDLr$^{-/-}$ mice was investigated via a spleen cell proliferation assay. Splenocytes isolated from naive LDLr$^{-/-}$ mice were incubated with several concentrations of oxLDL or MDA-LDL. Low concentrations of oxLDL, 1 and 5 μg/ml, resulted in a 1.53$\pm$0.17 ($P<0.05$) and 2.52$\pm$0.23 ($P<0.001$) fold increase in proliferation, respectively (Figure 2.1A). In case of MDA-LDL, incubation of splenocytes with 1, 5 and 10 μg/ml of MDA-LDL, resulted in a 1.62$\pm$0.15 (ns), 2.90$\pm$1.15 ($P<0.05$) and 4.41$\pm$0.89 ($P<0.01$) fold increase in proliferation, respectively (Figure 2.1B). OxLDL was toxic at concentrations $>$10 μg/ml, whereas MDA-LDL was only toxic at concentrations $>$100 μg/ml (data not shown). In all experiments conA, a general pan T cell activator, induced a $>$50-fold increase in proliferation (data not shown).

In addition we determined whether the T cell response to modified LDL can be modulated *in vivo*. LDLr$^{-/-}$ mice were immunized by intraperitoneal injection of 100 μg of oxLDL or MDA-LDL in combination with the adjuvant DDA. Two weeks thereafter, the mice were euthanized and isolated splenocytes were

incubated with oxLDL or MDA-LDL. OxLDL induced a higher proliferation at 1 and 5 μg/ml of 2.89±0.29 and a 7.25±0.81 fold, respectively, compared with the controls (P<0.01) (Figure 2.1C). Incubation of splenocytes isolated from MDA-LDL immunized mice with 1, 5 and 10 μg/ml MDA-LDL resulted in a 2.07±0.82 (ns), 3.94±0.41 (P<0.01) and 6.17±1.50 (P<0.05) fold increase in proliferation (Figure 2.1D).
A flow cytometric analysis was performed on the proliferating cells to determine the cell type responsible for the cell proliferation. The amount of $CD3^+$ T cells increased significantly from 34.5±1.5% to 46.9±2.4% when splenocytes were incubated with 5 μg/ml of oxLDL (Figure 2.1E; P<0.05). The amount of macrophages was not affected by the incubation with oxLDL (data not shown).

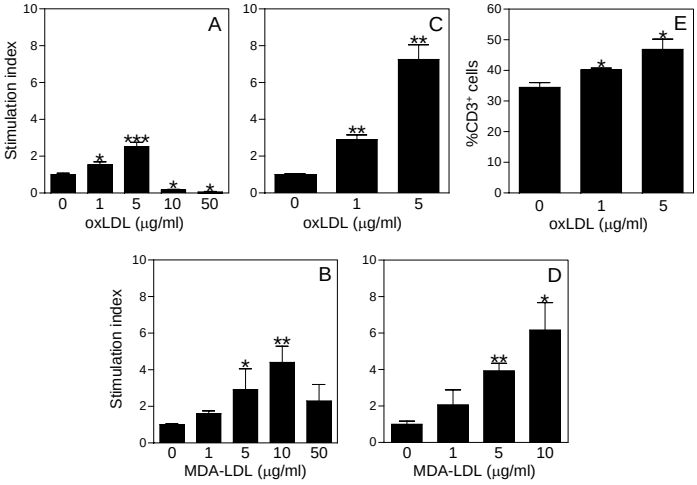

Figure 2.1: Spleen cell proliferation in response to oxLDL and MDA-LDL. $LDLr^{-/-}$ mice were immunized via one i.p. injection with 100 μg of oxLDL or MDA-LDL. Two weeks thereafter, splenocytes were isolated from naive and immunized $LDLr^{-/-}$ mice and cultured with oxLDL (A and C, respectively) or MDA-LDL (B and D, respectively) for 24 hrs. In the control situation, splenocytes from naive or immunized mice were cultured in absence of oxLDL or MDA-LDL. The amount of proliferation was measured by incorporation of ^{3}H-thymidine, which was added for the final 16 hours of the assay. Data are shown as the stimulation index (S.I.) ± SEM. The S.I. is defined as the ratio of the mean counts per minute of triplicate cultures with antigen to the mean counts per minute in culture medium without antigen. Graph E shows the percentage of $CD3^+$ cells (mean±SEM) after proliferation of the splenocytes with 1, 5 and 10 μg/ml of oxLDL. $^*P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001

Effect of oral tolerance induction to oxLDL and MDA-LDL on the initiation of atherosclerosis

To determine the effect of oral tolerance induction to oxLDL and MDA-LDL on atherosclerotic plaque initiation, atherosclerosis was induced after oral administration of oxLDL and MDA-LDL to $LDLr^{-/-}$ mice. Mice were put on a Western-type diet for one week and subsequently, oral tolerance was induced by oral administration of 30 μg of oxLDL or MDA-LDL. Treatment was repeated every other day to four times in total. After tolerance induction, the Western-type diet feeding was continued and one-week later mice were equipped with collars around both carotid arteries to induce atherosclerosis.[29] During the experiment

total plasma cholesterol levels were not significantly different between the groups and increased from 679±64 mg/dl (before diet) to 1554±140 mg/dl in control mice, to 1755±146 mg/dl in oxLDL treated mice and to 1718±77 mg/dl in MDA-LDL treated mice. Six weeks after collar placement atherosclerotic plaque formation was analyzed after a hematoxylin-eosin staining of cryosections of the carotid arteries (Figure 2.2A, 2.2B). Oral feeding of LDLr$^{-/-}$ mice with SOD had no effect on plaque size as compared to PBS (data not shown). Oral tolerance induction to oxLDL resulted in a significant 71.2% reduction in plaque area (Figure 2.2C; 20768±5964 versus 6046±1941 μm^2; P<0.05) when compared to control mice. Furthermore, a beneficial 55.3% reduction in intima/lumen ratio (Figure 2.2D; 0.25±0.06 versus 0.11±0.03; P<0.05) and a 72.1% reduction in intima/media ratio (data not shown; 0.75±0.23 versus 0.21±0.04; P<0.05) was observed. In case of MDA-LDL, oral tolerance induction did not result in a significant effect on plaque size (Figure 2.2E; 23314±8016 versus 37327±13137 μm^2; P=0.41) and also no significant effect on intima/lumen ratio (Figure 2.2F; 0.29±0.12 versus 0.50±0.16; P=0.34) and intima/media ratio (data not shown; 0.42±0.15 versus 1.12±0.34 in the MDA-LDL-treated group; P=0.12) was observed. In a second independent experiment the effect on plaque formation in the aortic root was determined at 8 weeks of diet. Oral treatment with oxLDL (Figure 2.3B) resulted in a significant 29.9% reduction in plaque size (Figure 2.3C; 669243±56643 versus 469288±27950 μm^2; P<0.01) when compared with control-treated mice (Figure 2.3A). Immunohistochemical analysis of the plaques of both experiments showed that oral tolerance induction to oxLDL and MDA-LDL had no effect on the relative macrophage and smooth muscle cell content when compared to plaques of control mice (data not shown).

Effect of oral tolerance induction to oxLDL on progression of atherosclerosis

Next we determined the effect of tolerance induction to oxLDL on the progression of atherosclerosis. To this end, LDLr$^{-/-}$ mice were put on a Western-type diet for 10 weeks, which resulted in the initial formation of plaques in the aortic root. At that time point oral tolerance to oxLDL was induced (30 μg of oxLDL, 4 times) and subsequently the LDLr$^{-/-}$ mice were kept on a Western-type diet for another 7 weeks. During the experiment total plasma cholesterol levels increased from 503±67 to 2727±243 mg/dl and 2422±366 mg/dl in the control and oxLDL-treated mice, respectively (no significant difference). After 7 weeks, the mice were sacrificed, cryosections of the aortic root of control-treated (Figure 2.4A) and oxLDL-treated (Figure 2.4B) mice were stained with Oil-red-O and atherosclerotic plaque formation in the aortic root was analyzed. OxLDL-treated mice showed a modest, but significant 24.3% reduction in plaque size at the aortic root (Figure 2.4C; 715523±56365 μm^2 versus 541353±55239 μm^2; P<0.05) as compared to control-treated mice. The effect of oral tolerance induction to oxLDL is more impressive when it is taken into account that the size of the plaques at the time of tolerance induction was 300000 μm^2 in a third group of mice. Subtraction of the lesion size at the start of the oral feeding establishes that oxLDL treatment led to a 42.4% reduction in plaque progression (Figure 2.4D). Interestingly, no effect of tolerance induction to oxLDL was observed on the relative macrophage and

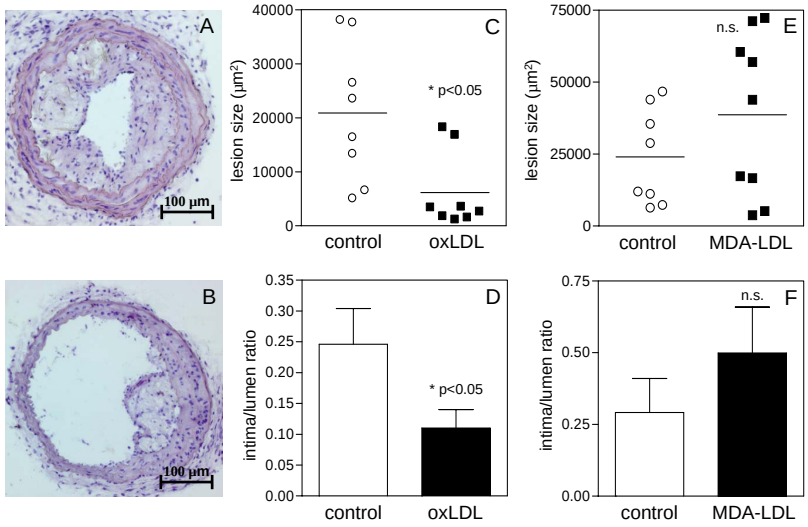

Figure 2.2: Effect of oral tolerance induction to oxLDL on atherosclerotic plaque formation in collar induced atherosclerosis in the carotid artery LDLr$^{-/-}$ mice were fed oxLDL or MDA-LDL four times before atherosclerosis was induced by collar placement around both carotid arteries. Six weeks after collar placement the mice were killed and the carotid arteries of control-treated (A), oxLDL-treated (B) and MDA-LDL-treated (not shown) mice were sectioned and stained with hematoxylin-eosin. The lesions were quantified by computer-assisted morphometric analysis and the plaque size and intima/lumen ratio of oxLDL-treated (C and D, respectively) and MDA-LDL-treated mice (E and F, respectively) were determined. * $P<0.05$

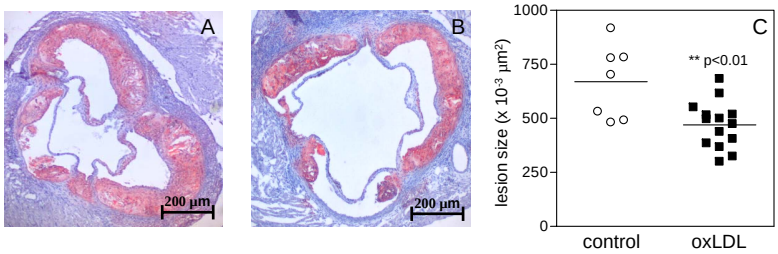

Figure 2.3: Effect of oral tolerance induction to oxLDL on atherosclerotic plaque formation in the aortic root LDLr$^{-/-}$ mice were fed oxLDL four times before atherosclerosis was induced by feeding a Western-type diet. After eight weeks of diet mice were killed and the aortic roots of control-treated (A) and oxLDL-treated (B) mice were sectioned and stained with Oil-red-O and hematoxylin. The lesions were quantified and the plaque size was determined (C). ** $P<0.01$

smooth muscle cell content in the plaque (data not shown).

Effect of oral tolerance induction to oxLDL on CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells

To determine whether oral tolerance induction to oxLDL was associated with a change in regulatory T cell levels, a flow cytometric analysis was performed. CD4$^+$CD25$^+$Foxp3$^+$ cells are normally present in low numbers in spleen (0.8±0.2%), mesenteric lymph nodes (3.0±0.4%), Peyer's patches (1.7±0.4%) and blood (1.7±0.1%) of control LDLr$^{-/-}$ mice. Two and four days after the

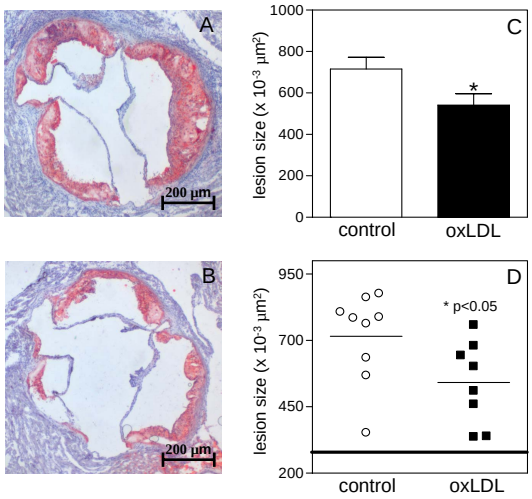

Figure 2.4: Oral tolerance induction to oxLDL attenuates atherosclerotic plaque progression in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were fed a Western-type diet for 10 weeks before oxLDL was administered orally 4 times. After oral tolerance induction the mice were kept on a Western-type diet for 7 more weeks. The aortic roots of control-treated (A) and oxLDL-treated (B) mice were sectioned and stained with Oil-red-O and hematoxylin. Lesions at the aortic root were quantified by computer-assisted morphometric analysis and the plaque size (C) was determined. Graph D shows a dot plot of lesion size in all mice. The bar at $0.30{\cdot}10^6$ μm^2 represents the lesion size of mice after 10 weeks of diet without oral treatment. $^*P<0.05$

fourth and last oral feeding of oxLDL, the number of $CD4^+CD25^+Foxp3^+$ cells increased significantly to 1.3±0.1% and 1.6±0.2% in the spleen, respectively (Figure 2.5A; $P<0.05$) and to 5.2±0.2% and 5.8±0.6% in mesenteric lymph nodes, respectively (Figure 2.5B; $P<0.01$). No significant changes were seen in the Peyer's patches and blood. To determine if the effect was long lasting, mice were sacrificed 14 days after the last oral feeding. $CD4^+CD25^+Foxp3^+$ cells were still increased in spleen and mesenteric lymph nodes (1.4±0.1% ($P<0.05$) and 5.4±0.3% ($P<0.01$), respectively) after oral feeding of oxLDL (Figure 2.5A, 2.5B), whereas MDA-LDL did not affect the number of $CD4^+CD25^+Foxp3^+$ cells (not shown). To determine the effect of induction of $CD4^+CD25^+Foxp3^+$ cells on cytokine production, mesenteric lymph nodes of control, oxLDL, and MDA-LDL-treated mice were re-stimulated with 5 μg/ml oxLDL or MDA-LDL *in vitro*. We observed that oxLDL only induced a significant 6-fold increase in TGF-β in lymph node cells from oxLDL tolerant mice, but not in control pretreated mice, whereas MDA-LDL was unable to induce TGF-β in MDA-LDL pretreated or control mice (not shown). The IL-10 and IFN-γ levels were in any of the experiments below the detection limits.

Expression of regulatory T cell markers in atherosclerotic plaques

We analyzed the expression of CD25 and Foxp3 in atherosclerotic plaques in the carotid arteries. After treatment with oxLDL (n=14) and 8 weeks of Western-type diet feeding, the relative mRNA expression of Foxp3, CTLA-4 and CD25 was significantly upregulated in the atherosclerotic plaque when compared with

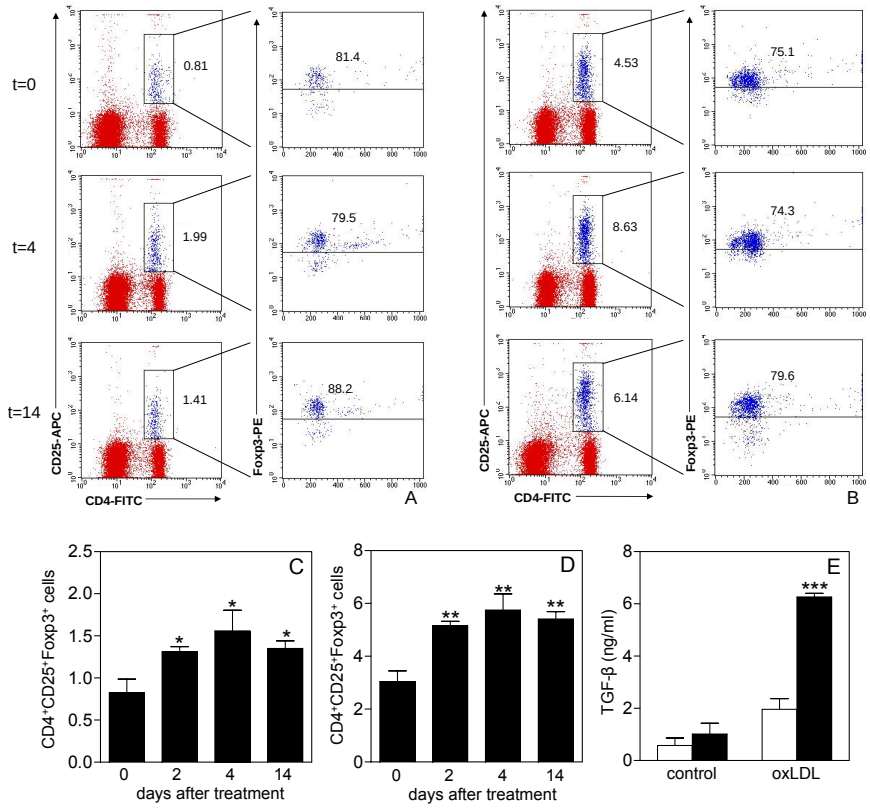

Figure 2.5: Effect of oral tolerance induction to oxLDL on $CD4^+CD25^+Foxp3^+$ cells in spleen and MLNs. $LDLr^{-/-}$ mice were fed PBS or oxLDL 4 times and were killed at day 2, 4 and 14. The dot plots show representative examples of lymphoid cells isolated from the spleen (A) and mesenteric lymph nodes (B) stained for CD4 and CD25 (left panels). The right panel of dot plots shows the percentage of $Foxp3^+$ cells within the $CD4^+CD25^+$ population. Graph C represents the percentage of $CD4^+CD25^+Foxp3^+$ cells in the spleen, graph D the percentage of $CD4^+CD25^+Foxp3^+$ cells in the mesenteric lymph nodes (mean±SEM). (E) TGF-β production by mesenteric lymph node cells isolated 14 days after treatment from control and oxLDL-treated mice and restimulated with PBS (open bars) or oxLDL (closed bars) *in vitro*. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (compared to all other bars)

control mice (n=8). Foxp3 showed a 1.5-fold increase, CTLA-4 a 1.7-fold increase and CD25 a 2.2-fold increase (Figure 2.6; $P<0.05$).

Influence of oral tolerance induction to oxLDL on IgG patterns

OxLDL-specific IgG1 and IgG2a levels in serum were determined at the end of the experiment on the initiation of atherosclerosis. No detectable differences in IgG1 and IgG2a levels were observed (Figure 2.7A, 2.7B, respectively) and no difference in the IgG1/IgG2a ratio in control and oxLDL-treated mice was observed (Figure 2.7C).

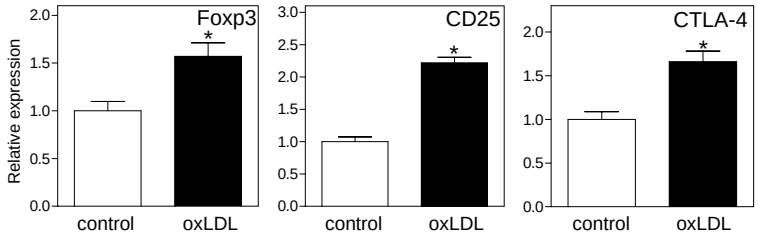

Figure 2.6: Expression of regulatory T cell markers in oxLDL-treated and control mice. mRNA was isolated from carotid arteries of control (n=8) and oxLDL-treated (n=14) mice and the mRNA levels of Foxp3, CTLA-4 and CD25 were quantitatively determined and expressed relative to 36B4. $*P<0.05$

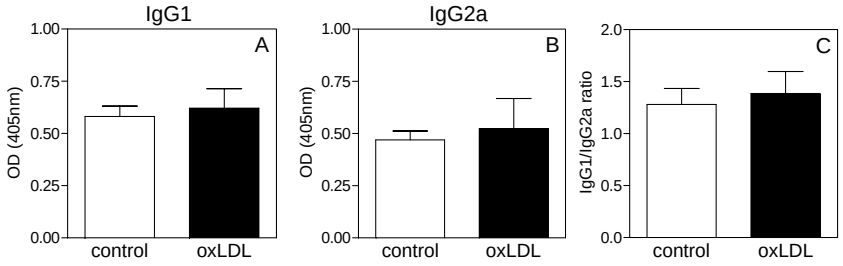

Figure 2.7: Effect of tolerance to oxLDL on oxLDL-specific IgG1 and IgG2a levels. LDLr$^{-/-}$ mice were treated orally with PBS or oxLDL and serum levels of oxLDL-specific IgG1 (A) and IgG2a (B) were measured using a capture enzyme-linked immunosorbent assay. Values are mean OD(405) values ± SEM. Graph C represents the effect of oral tolerance induction to oxLDL on the IgG1/IgG2a ratio.

Discussion

One of the first events in atherosclerosis is the oxidative modification of LDL and the subsequent uptake of oxLDL by macrophages. Epitopes from oxLDL, such as apolipoprotein B-100 peptides,[30] and oxidized phosphorylcholine-containing phospholipids,[31] can be presented by antigen presenting cells and result in T cell activation.[32] Autoreactive T cells specific for oxLDL epitopes have been found in human atherosclerotic plaques,[1] and in atherosclerotic lesions in apoE$^{-/-}$ mice.[2] We now demonstrate that naive LDLr$^{-/-}$ mice already contain T cells specific for oxLDL and MDA-LDL as shown in spleen cell proliferation assays. In addition, we show that the *in vivo* response to oxLDL and MDA-LDL can be modified by immunization with oxLDL or MDA-LDL. The spleen cell proliferation showed an enhanced proliferation (3-7-fold) in the immunized mice when compared to the naive mice.

It is well known that the T cells in atherosclerotic lesions, reactive to several antigens such as oxLDL and heat shock proteins, mainly produce Th1 cytokines resulting in a disturbed balance between Th1 and Th2 cytokines. Several studies show that via Th1 cytokine inhibition the extent of atherosclerosis can be reduced.[28,33–35] The other way around, stimulation of the Th2 cytokine production can attenuate atherosclerosis.[29,36] Although restoration of the imbalance between Th1 cells and Th2 cells may be effective in atherosclerosis, some debate on the beneficial role of Th2 cells in atherosclerosis exists:

experiments with IL-4, a Th2 cytokine, show that IL-4 may be pro-atherogenic.[33] Mallat et al. hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and/or Th2) and regulatory T cells (Tregs) specific for 'altered' self and non-self antigens.[37] Recently, Ait-Oufella et al. showed that regulatory T cells play an important role in controlling the development of atherosclerosis.[38] A possible mechanism to achieve a beneficial shift in the balance between pathogenic T cells and regulatory T cells is mucosal tolerance induction. The regulatory T cells induced via nasal tolerance, Tr1 cells, mainly produce IL-10 and regulatory T cells induced via oral tolerance, Th3 cells, mainly produce TGF-β.[39] Besides Tr1 and Th3 cells, mucosal tolerance induction can also lead to activation of $CD4^+CD25^+Foxp3^+$ cells. Foxp3 is known as an exclusive marker for natural regulatory T cells.[40] The regulatory function of these cells is mediated by cell contact and surface-bound TGF-β and CTLA-4.[21]

In our current study we show that oral tolerance induction to oxLDL can both attenuate atherosclerosis in an early stage and in an advanced stage. A relative low dose of oxLDL (30 μg, four times) significantly attenuated early atherosclerotic lesion formation in the carotid arteries by 71.2% and at the aortic root by 29.9%. The effect on lesion initiation is reflected in the intima/lumen ratio (55.3% reduction) and the intima/media ratio (72.1% reduction) of carotid arteries. Our results are in line with studies that showed that oral or nasal tolerance induction to HSP65 and β2-glycoprotein I may be a useful treatment for atherosclerosis.[22–24] In our current study also a significant 24.3% reduction in advanced atherosclerotic lesion size was observed. Taken the initial lesion size into account a 42.4% reduction in plaque progression is obtained. In line with the studies on HSP65 and β2-glycoprotein I, no effect on the relative macrophage and collagen content of the plaque was seen in both experiments.[22,24] We have previously shown that overexpression of IL-10, another way to modulate the inflammatory process in atherosclerosis, largely reduced (62.2%) the atherosclerotic lesion formation without any effect on macrophage, collagen and SMC content of the plaque.[29]

Besides oxLDL, MDA-LDL was also used in this study as antigen for oral tolerance induction. Surprisingly, no significant effect on atherosclerotic plaque area was seen after oral tolerance induction with 30 μg of MDA-LDL in $LDLr^{-/-}$ mice. The $LDLr^{-/-}$ mice treated orally with MDA-LDL showed a 60.8% (P=0.41) increase in plaque area and a 71.1% (P=0.34) increase in intima/lumen ratio.

We also explored the possible effect of oral tolerance induction on the antibody isotype distribution. No significant alterations in oxLDL-specific IgG1 and IgG2a levels were detected and consequently also no effect on the IgG1/IgG2a ratio was observed, suggesting that the Th1/Th2 ratio in the mice was not altered by tolerance induction.

The lack of an effect on oxLDL-specific antibodies demonstrate that the suppressing effects shown after oral tolerance induction are not caused by an effect on the humoral immune response. In the majority of the studies and in all studies on atherosclerosis the exact mechanism behind oral tolerance is still unclear. Increasing levels of IL-10 and TGF-β after oral tolerance induction are dedicated to a stimulated development of adaptive immune cells.[24] Zhang et al. found an increased amount of $CD4^+CD25^+$ cells after feeding mice with

OVA. These "regulatory" T cells were declared to be responsible for the high levels of IL-10 and TGF-β.[41] Recently, $CD4^+CD25^+$ cells with Foxp3 expression were found to be immuno-suppressive. We demonstrate that oxLDL tolerance induction significantly increases the number of $CD4^+CD25^+Foxp3^+$ cells for up to two weeks in the spleen and the mesenteric lymph nodes. Since these regulatory T cells exert their suppressive effect via cell-cell contact and surface-bound TGF-β, we determined the TGF-β production in the mesenteric lymph nodes. Lymph node cells from oxLDL tolerant mice produced 6-fold higher levels of TGF-β upon re-stimulation with oxLDL than cells from control mice, indicating the induction of TGF-β producing regulatory T cells.
In addition, analysis of mRNA expression levels showed that oxLDL tolerance induction increased the expression of Foxp3, CTLA-4 and CD25 within the plaque, clearly indicating the presence of regulatory T cells within the lesions upon tolerance induction. The profile of gene expression upon oxLDL tolerance induction, together with the induction of Foxp3 in spleen and lymph nodes and concurrent oxLDL specific TGF-β production in the absence of IL-10 and IFN-γ production highly suggest that $CD4^+CD25^+Foxp3^+$ Tregs rather than Tr1 or Th3 are responsible for the observed effects of oxLDL tolerance induction on atherosclerosis. The absence of induction of Tregs by MDA-LDL and the absence of TGF-β induction in lymph node cells of MDA-LDL pretreated cells by MDA-LDL may explain the absence of an regulatory effect of oral treatment with MDA-LDL.
It may be speculated that oxLDL specific Tregs induced by oral tolerance regulate the action of oxLDL specific $CD4^+$ T cells within the plaque in the following way: oxLDL specific $CD4^+$ T cells are within the plaque activated by oxLDL presenting APCs, which may lead to the expression of TGF-β receptor II (TβRII) by these cells. OxLDL specific Tregs also recognize oxLDL presented by the APCs and via the enhanced production of TGF-β, Tregs can modulate the action of oxLDL specific $CD4^+$ T cells.[42,43] TGF-β-TβRII interaction leads to the activation of a Smad-dependent pathway, resulting in a blockade of IL-2 production[43,44] and a reduced proliferation of oxLDL-specific T cells.
In conclusion we describe that $LDLr^{-/-}$ mice can be tolerized to oxLDL which results in an attenuation of both early an advanced atherosclerotic lesions. The mechanism underlying this effect can be dedicated to the induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells which counteract within the plaque the oxLDL-specific $CD4^+$ T cells. These results are promising and prove that the mechanism of oral tolerance induction could be an effective treatment for atherosclerosis.

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Chapter 3

Induction of oral tolerance to HSP60 or an HSP60-peptide activates T cell regulation and reduces atherosclerosis

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Abstract
HSP60-specific T cells contribute to the development of the immune responses in atherosclerosis by producing proinflammatory cytokines. This can be dampened by regulatory T cells (Tregs) which are activated via oral tolerance induction. In this study we explored the effect of oral tolerance induction to HSP60 and the peptide HSP60(253-268) on experimental atherosclerosis. HSP60 and HSP60(253-268) were administered orally to $LDLr^{-/-}$ mice prior to induction of atherosclerosis and oral tolerance induction resulted in a significant 80.7% and 81.3% reduction ($P<0.05$) in plaque size in the carotid arteries, respectively, and in a 27.4% reduction in plaque size at the aortic root in HSP60-treated mice. Reduction in plaque size correlated with an increase in the number of $CD4^+CD25^+Foxp3^+$ Tregs in several organs and also an increased expression of Foxp3, CD25 and CTLA-4 in the atherosclerotic lesion was observed in HSP60-treated mice. In addition, an increased production of IL-10 and TGF-β by mesenteric lymph node cells in response to HSP60 was observed, while splenocytes from HSP60-treated mice proliferated much lower in response to HSP60 when compared with PBS-treated mice. In conclusion, oral tolerance induction to HSP60 and a small HSP60-peptide leads to an increase in the number of specific $CD4^+CD25^+Foxp3^+$ Tregs, resulting in a decrease in plaque size as a consequence of increased production of IL-10 and TGF-β. We conclude that these beneficial results of oral tolerance induction to HSP60 and HSP60(253-268) may provide new therapeutic approaches for the treatment of atherosclerosis.

Introduction

Heat shock proteins (HSPs) are a family of highly conserved proteins with various functions in normal and stressful situations. Expression of HSPs on endothelial cells and macrophages[1,2] can be induced by several stress factors, such as fluid shear stress,[3] oxidized lipoproteins[4] and cytokines.[2] Under these circumstances, HSPs repair or prevent degradation of denaturated proteins and increase the cell's ability to survive stressful stimuli.[5,6] HSPs such as HSP60 are also involved in inflammatory diseases, probably resulting from their raised expression in cells exposed to proinflammatory mediators.[7,8] In human atherosclerotic lesions,[9] enhanced HSP60 expression has been detected. In addition, patients with atherosclerosis show an elevated concentration of HSP60-specific antibodies in serum,[2] and T cell clones with self-HSP60 reactivity have been detected within the atherosclerotic plaques.[10] This may be related to initial immune responses against bacterial heat shock proteins which are highly homologous to HSPs in various other species including men, rats and mice.[11] It is possible that the HSP60-specific antibodies contribute to endothelial damage and the inflammatory response in the vessel wall accelerating atherosclerosis.[12]
The autoimmune process in atherosclerosis is characterized by a T cell response to different autoantigens, e.g. oxidized LDL[13], glycoproteins[14] and HSPs[15]. HSP60-specific T cells are mainly of a Th1 phenotype, producing pro-atherogenic cytokines, such as IFN-γ, IL-12 and TNF-α and causing a disturbed balance between Th1 and Th2 cytokines in atherosclerosis.[16] For a long time, this disturbed balance was regarded as the cause of the ongoing inflammation in atherosclerosis. Recent publications however suggest that Tregs play an important role in prevention of Th1 mediated autoimmune diseases such as multiple sclerosis,[17] diabetes mellitus[18] and atherosclerosis.[19] Mallat et al. hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and Th2) and Tregs (Tregs) specific for 'altered' self and non-self antigens (e.g. oxidized phospholipids, heat shock proteins).[20]
One way to increase the number of antigen specific Tregs is "low dose" oral tolerance induction. This method is already used as a treatment in animal models for Th1 mediated autoimmune diseases such as multiple sclerosis,[21,22] rheumatoid arthritis[23,24] and type I diabetes.[25,26] Initial studies also show that oral tolerance induction to β2-glycoprotein I[27] and HSP65[28,29] results in the suppression of early atherosclerosis. However, these studies do not show the involvement of Tregs. We describe in a recent study an increase in the number of $CD4^+CD25^+Foxp3^+$ cells after oral tolerance induction to oxidized LDL (oxLDL)[30] and a subsequent reduction in plaque size. These $CD4^+CD25^+Foxp3^+$ cells form a class of Tregs that may either be natural Tregs which act via cell-cell contact via surface-bound TGF-β[31] or cytotoxic T lymphocyte-associated antigen-4 (CTLA-4)[32] or they may be adaptive Tregs operating via the secretion of TGF-β.
In the present study we demonstrate that induction of "low dose" oral tolerance to HSP60 and a peptide based on the highly conserved 253-268 sequence of mycobacterial HSP60 (HSP60(253-268)) attenuates atherosclerosis. The regulatory effect on atherosclerosis is explained by an increased number of

$CD4^+CD25^+Foxp3^+$ Tregs in both lymphoid organs and the atherosclerotic lesion. This is accompanied by an increase in HSP60-specific TGF-β and IL-10 production in mesenteric lymph node cells. In addition, tolerance induction to HSP60 reduces the proliferation of splenocytes in response to HSP60.

Methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male $LDLr^{-/-}$ mice were obtained from the Jacksons Laboratory. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a 'Western-type' diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). All mice used were 10-12 weeks of age. Diet and water were administered *ad libitum*.

Antigens and adjuvant

Dimethyl dioctadecyl ammonium bromide (DDA; Sigma Diagnostics, MO), used as adjuvant, was dissolved in phosphate buffered saline (PBS) and 100 μg was mixed with 100 μg of the antigen (HSP60, HSP60(253-268) or HSP70(111-125)) before immunization. Purified recombinant HSP60 of *Mycobacterium bovis* bacillus Calmette-Gurin was kindly provided by J.D.A. van Embden (National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands). HSP60(253-268) based on the sequence of mycobacterial HSP60 aa 253-268 (NH_2-EGEALSTLVVNKIRGT-amide), was made by regular peptide synthesis (FMOC protection). Another peptide HSP70(111-125) was based on a partially conserved (human, rat, mouse) sequence of the HSP70 peptide aa 111-125 (NH_2-ITDAVITTPAYFNDA-amide).[33]

Immunizations

$LDLr^{-/-}$ mice were immunized via one i.p. injection with PBS or 100 μg of HSP60, HSP60(253-268) or HSP70(111-125). The antigens were dissolved in 200 μl of PBS containing 100 μg DDA. After 14 days the spleens were dissected and used in the proliferation assay described below.

Spleen Cell Proliferation Assay

Spleens from either naive (n=3 or 12), immunized (n=3) or oral treated mice (n=12) were dissected and squeezed through a 70 μm cell strainer (Falcon, The Netherlands). The erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). The splenocytes were cultured for 48 hours in triplicate at $2{\cdot}10^5$ cells per well of a 96-wells round-bottom plate in the presence or absence of different concentrations of HSP60, HSP60(253-268) or HSP70(111-125). RPMI 1640 (with L-Glutamine, 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml

streptomycin (all from BioWhittaker Europe)) was used as culture medium. Concanavalin A (Con A; Sigma-Diagnostics, MO) (2 μg/ml) was used as a positive control. Cultures were pulsed for an additional 16 hours with [6-^{3}H]-thymidine (1 μCi/well, sp. act. 24 Ci/mmol; Amersham Biosciences, The Netherlands). The amount of [6-^{3}H]-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The magnitude of the proliferative response is expressed as stimulation index (SI) defined as the ratio of the mean counts per minute of triplicate cultures with antigen to the mean counts per minute in culture medium without antigen.

Induction of atherosclerosis

To determine the effect of oral tolerance induction on the initiation of atherosclerosis, atherosclerosis was induced in LDLr$^{-/-}$ mice. The mice were put on a Western-type diet three weeks prior to surgery. Atherosclerosis was induced by placement of perivascular collars, prepared from elastic tubing (0.3 mm inside diameter; Dow Corning, Midland, Michigan), around both carotid arteries (method described by von der Thüsen et al.[34]). During the experiment, the diet response was followed by measuring the cholesterol and triglyceride levels in serum of these mice. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard.

Oral tolerance induction

After one week of Western-type diet and two weeks prior to collar placement, the LDLr$^{-/-}$ mice were treated 4 times over a period of 8 days with intragastrically administered antigens. Before each intragastrical administration, the animals were deprived of food but not water for 16 hours. To prevent degradation of the administered antigen, 2 mg of soybean trypsin inhibitor (STI, Sigma-Diagnostics, MO) was administered intragastrically. Ten minutes after the STI administration, the control group received 100 μl of PBS (n=7). The other mice received 30 μg of HSP70(111-125) (n=6), HSP60 (n=6) or HSP60(253-268) (n=7). All antigens and STI were diluted and dissolved in physiological saline (0.9% NaCl) prior to injection. After administering the antigens intragastrically, the mice were kept on Western-type diet for another week before collars were placed.

Plaque analysis

Six weeks after collar placement the mice were euthanized and exsanguinated by femoral artery transsection. The mice were perfused and fixated through the left cardiac ventricle with PBS and FormalFixx (Thermo Shandon, Pittsburgh, PA) for 30 min. Common carotid arteries and the heart with the aortic root were removed for analysis as described by von der Thüsen et al.[34] The arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and proximally of the place of collar occlusion 5 μm sections were made on a Leica CM 3050S Cryostat (Leica Instruments, UK). These cryosections were stained with

hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). 10 μm section were made of the aortic root and these sections were stained with Oil-red-O and hematoxylin. Plaque areas and intima/lumen ratios were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Flow cytometric analysis

For the detection of $CD4^+CD25^+Foxp3^+$ T cells, a three color flow cytometry was performed. 4 and 14 days after oral treatment with HSP60, spleen, mesenteric lymph nodes, Peyer's patches, and blood were isolated from HSP60-treated and untreated mice (n=5). Mononuclear cells were isolated using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. Cells were subsequently stained with FITC-conjugated anti-CD4 (0.125 μg/sample) and APC-conjugated anti-CD25 (0.06 μg/sample) mAb (eBioscience, Belgium) for 30 min. Cells were then fixed and permeabilized for 16 hrs with Fixation/Permeabilization solution according to the suggested protocol (eBioscience, Belgium). Subsequently, the cells were stained with PE-conjugated anti-Foxp3 (0.2 μg/sample) (eBioscience, Belgium) for 30 min. Cells were analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Cytokine assays

Mesenteric lymph nodes were isolated from untreated (n=5) and HSP60-treated mice (n=5) 14 days after oral treatment with HSP60. The lymph nodes were squeezed through a cell strainer and the cells were cultured at $1{\cdot}10^6$ cells per well of a 24-wells plate in the presence or absence of 20 μg/ml HSP60. Culture supernatants were harvested after 48 hours of incubation. IL-10, IFN-γ (both from eBioscience, Belgium) and TGF-β (Bender MedSystems, Austria) concentrations were determined by enzyme-linked immunosorbent assays (ELISA) according to the manufacturers suggestions.

Real-time PCR assays

Carotid arteries from control and HSP60-treated mice were isolated and mRNA was extracted using the guanidium isothiocyanate (GTC) method and reverse transcribed (RevertAid M-MuLV reverse transcriptase). Quantitative gene expression analysis was performed on an ABI PRISM 7700 sequence detector (Applied Biosystems, CA) using SYBR green technology. The following primer pairs were used: 5'-GGAGCCGCAAGCTAAAAGC-3' and 5'-TGCCTTCGTGCCCACTGT-3' for Foxp3; 5'-CTTATATTGCAAATGTGGCACAATC-3' and 5'-ATCAATCATCAGTGGGACAATCTG-3' for CD25; 5'-CGAGGTCCTGCACCAACTG-3' and 5'-TCCATCACCATCGGTTTATGC-3' for CTLA4. Acidic ribosomal phosphoprotein PO (36B4) was used as the endogenous reference gene and detected using the primers 5'-GGACCCGAGAAGACCTCCTT-3' and 5'-GCACATCACTCAGAATTTCAATGG-3'.

Detection of anti-HSP60 antibodies

HSP60 (10 μg/ml) dissolved in a $NaHCO_3/Na_2CO_3$ buffer (pH 9.0) was coated. Measurement of IgG1, IgG2a and IgM levels in serum was performed using an ELISA Ig detection kit (Zymed Laboratories, CA) conform the manufacturer's protocol and appropriate controls were performed.

Statistical analysis

All data are expressed as mean $\pm$ SEM. The two-tailed student's t-test was used to compare proliferative responses to antigens, FACS data, cytokine levels, mRNA expression and atherosclerotic parameters between the different groups. P-values less than 0.05 are considered to be statistically significant.

Results

T cells specific for HSP60 and HSP60(253-268) epitopes are present in LDLr$^{-/-}$ mice

Because of the important role of HSP60-specific T cells in atherosclerosis, we first investigated the presence of T cells specific for HSP60, HSP60(253-268) or HSP70(111-125) epitopes in the LDLr$^{-/-}$ mice. Splenocytes were isolated out of naive LDLr$^{-/-}$ mice and were incubated with several concentrations of the HSP epitopes. Incubation with 5 μg/ml HSP60 or HSP60(253-268) had no effect on naive splenocytes, while incubation with 20 μg/ml HSP60 or HSP60(253-268) resulted in a 2.70$\pm$0.42 and 2.04$\pm$0.35 fold increase in proliferation, respectively (Figure 3.1A and B; $P<0.05$). HSP70(111-125) did not stimulate proliferation of the splenocytes (data not shown). In all experiments ConA, a general pan T cell mitogen, was used as a positive control, and incubation of splenocytes with 2 μg/ml of ConA resulted in a more than 50-fold increase in proliferation (data not shown).
To determine whether the T cell response to HSP-epitopes can be induced in vivo we immunized LDLr$^{-/-}$ mice by an intraperitoneal injection of 100 μg of HSP60, HSP60(253-268) or HSP70(111-125) using DDA as adjuvant. After two weeks mice were killed, and isolated splenocytes from HSP60-immunized mice incubated with 5 and 20 μg/ml of HSP60 showed a 7.40$\pm$1.29 ($P<0.01$) and a 12.71$\pm$2.30 ($P<0.01$) fold increase in proliferation, respectively (Figure 3.1C). Incubation of splenocytes from HSP60(253-268)-immunized mice with 5 and 20 μg/ml HSP60(253-268) resulted in a 7.29$\pm$2.32 ($P<0.05$) and 9.26$\pm$2.58 ($P<0.01$) fold increase, respectively (Figure 3.1D). Incubation of splenocytes from HSP70(111-125)-immunized mice with 5 and 20 μg/ml HSP70(111-125) did not result in a significant effect on proliferation (Figure 3.1E).

Effect of oral tolerance induction to HSP60, HSP60(253-268) and HSP70(111-125) on atherosclerosis

Next we investigated the immunomodulatory effect of oral tolerance induction to these compounds on atherosclerosis. LDLr$^{-/-}$ mice were put on a Western-type diet for one week prior to oral administration of PBS or 30 μg of HSP60,

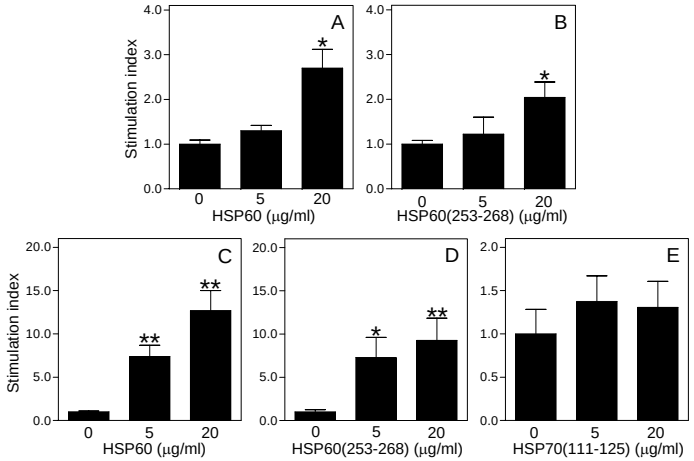

Figure 3.1: Spleen cell proliferation in response to HSP60 and HSP60(253-268). Splenocytes were isolated from naive LDLr$^{-/-}$ mice (A and B) and mice immunized via i.p. injection with 100 μg of HSP60, HSP60(253-268) or HSP70(111-125) (C,D and E). The naive and primed splenocytes were re-stimulated *in vitro* with HSP60 (A and C), HSP60(253-268) (B and D) or HSP70(111-125) (E) for 48 hours. Proliferation was measured by incorporation of ^{3}H-thymidine. Data are shown as the stimulation index (S.I.) $\pm$ SEM. The S.I. is the ratio of the mean cpm of cultures with antigen to the mean cpm of cultures without antigen. $^{*}P<0.05$, $^{**}P<0.01$.

HSP60(253-268) or HSP70(111-125). The oral treatment was given 4 times in total, every other day. Subsequently, mice were equipped with collars around both common carotid arteries and fed a Western-type diet. Six weeks thereafter, atherosclerotic plaque formation was analyzed. Representative hematoxylin-eosin stained cryosections of the carotid arteries of PBS, HSP70(111-125), HSP60, and HSP60(253-268)-treated mice are shown in figure 3.2A-D. No significant difference in plaque size was observed in LDLr$^{-/-}$ mice fed HSP70(111-125) (21181$\pm$5273 μm^2) compared to PBS-treated mice (20471$\pm$5273 μm^2). Oral administration of HSP60 (3959$\pm$582 μm^2) resulted in a significant 80.7% ($P<0.01$) reduction in plaque size when compared to PBS-treated mice. Oral tolerance induction to HSP60(253-268) (3419$\pm$460 μm^2) resulted in an 83.3% ($P<0.05$) reduction in plaque size (Figure 3.2E). Furthermore, the intima/lumen ratio was reduced significantly with 68.8% in the HSP60 treated mice ($P<0.05$; 0.082$\pm$0.007) and with 74.3% in the HSP60(253-268)-treated mice ($P<0.05$; 0.067$\pm$0.010) when compared to the PBS-treated mice (0.261$\pm$0.074) (Figure 3.2F). During the experiment total plasma cholesterol levels increased due to the Western-type diet, but no significant differences were detected between the different groups (Figure 3.2G). In addition, lesion development at the aortic root of PBS-treated mice (Figure 3.3A) and HSP60-treated mice (Figure 3.3B) was investigated. A 27.4% reduction in plaque size at the aortic root was observed in HSP60-treated mice (377000$\pm$37200 μm^2) when compared with PBS-treated mice (Figure 3.3C; 519000$\pm$44600 μm^2; $P<0.05$). Immunohistochemical analysis of all plaques showed that oral tolerance induction to HSP60 and HSP60(253-268) had no effect on the relative macrophage and smooth muscle cell content (data not shown). To determine the effect of tolerance induction to HSP60 on the HSP60-specific proliferation of splenocytes, splenocytes were isolated from PBS-treated

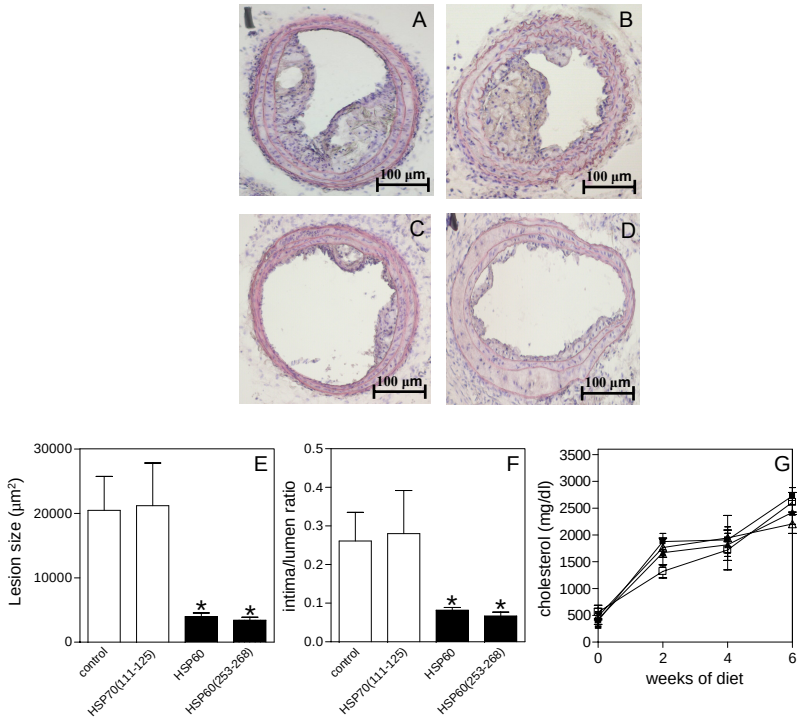

Figure 3.2: Oral tolerance induction to HSP60 and HSP60(253-268) attenuates plaque formation in collar induced atherosclerosis in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were fed PBS, HSP70(111-125), HSP60 or HSP60(253-268) four times before induction of atherosclerosis and six weeks thereafter mice were sacrificed and the carotid arteries of PBS-treated (A), HSP70(111-125)-treated (B), HSP60-treated (C) and HSP60(253-268)-treated (D) mice were sectioned and stained with hematoxylin-eosin. Lesions were quantified by computer-assisted morphometric analysis and plaque size (E) and intima/lumen ratio (F) were determined. During the experiment plasma cholesterol levels of PBS-treated (closed squares), HSP70(111-125)-treated (closed triangles), HSP60-treated (open squares) and HSP60(253-268)-treated (open triangles) mice were monitored (G). $^{*}P<0.05$

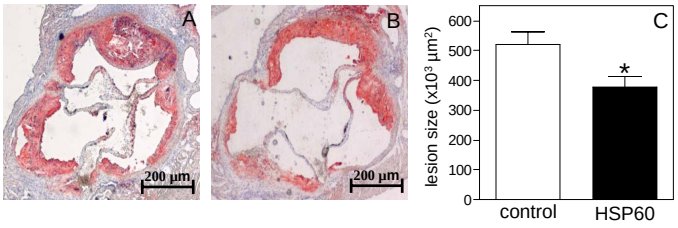

Figure 3.3: Oral tolerance induction to HSP60 reduces plaque formation at the aortic root in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were fed a Western-type diet and were treated intragastrically four times with PBS or HSP60 as in figure 2. After 8 weeks, sections of the aortic root of PBS-treated (A) and HSP60-treated (B) mice were stained with Oil-red-O and hematoxylin and subsequently lesions were quantified and plaque size was determined (C). Values are mean lesion size ± SEM. $^{*}P<0.05$

and HSP60-treated mice. The splenocytes were cultured with or without 5 and 20 μg/ml of HSP60. Splenocytes from PBS-treated mice respond to HSP60 with an increased proliferation; a stimulation index of 4.4±0.7 and 10.4±2.5 when

incubated with 5 and 20 μg/ml of HSP60, respectively. Mice orally treated with HSP60 showed a 56.8% and 68.2% reduction in the proliferative response to 5 and 20 μg/ml of HSP60, respectively (Figure 3.4; 1.9±0.2 and 3.3±0.4; $P<0.05$).

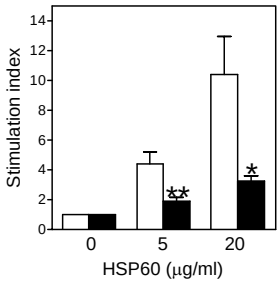

Figure 3.4: Oral tolerance induction to HSP60 reduces the proliferative response of splenocytes to HSP60. LDLr$^{-/-}$ mice in which atherosclerosis was induced by a combination of Western-type diet feeding and collar placement around both carotid arteries were treated 4 times intragastrically with PBS or HSP60 after one week. Seven weeks later, splenocytes of PBS-treated mice (white bars) and HSP60-treated mice (black bars) were cultured with or without 5 and 20 μg/ml of HSP60. The extent of proliferation is shown as stimulation index. Values are mean±SEM. $^{*}P<0.05$ $^{**}P<0.01$

Effect of oral tolerance induction to HSP60 on CD4^{+}CD25^{+}Foxp3^{+} Tregs

To evaluate whether oral tolerance induction to HSP60 was associated with a change in Tregs, flow cytometry analysis was performed. HSP60-treated LDLr$^{-/-}$ mice were sacrificed 4 and 14 days after the oral treatment. In untreated control mice, CD4^{+}CD25^{+}Foxp3^{+} T cells are present in low numbers in Peyer's patches (0.79±0.16%), blood (2.21±0.12%), spleen (0.80±0.07%) and mesenteric lymph nodes (3.82±0.25%). The dot-plots in figure 3.5A are one representative example of FACS analysis on CD4^{+}CD25^{+} cells (left panels) and Foxp3^{+} cells within the CD4^{+}CD25^{+} population (right panels) in mesenteric lymph nodes. 4 days after oral treatment with HSP60, the number of CD4^{+}CD25^{+}Foxp3^{+} T cells in the Peyer's patches and blood was increased significantly to 1.73±0.30% ($P<0.05$) and 2.86±0.21% ($P<0.01$), respectively, when compared to untreated mice (Figure 3.5B, upper part). No significant change was seen in the mesenteric lymph nodes (4.67±0.41%) and spleen (0.85±0.06%) (Figure 3.5B, lower part). 14 days after oral treatment, the number of CD4^{+}CD25^{+}Foxp3^{+} T cells in the Peyer's patches decreased again to 1.07±0.08% and was not significantly different from untreated mice whereas the number of CD4^{+}CD25^{+}Foxp3^{+} T cells in blood was still enhanced (2.81±0.20%, $P<0.01$) (Figure 3.5A, upper part), while in the mesenteric lymph nodes and spleen a significant increase to 5.36±0.10% ($P<0.01$) and 1.24±0.11% ($P<0.01$) was observed when compared with the situation without treatment (Figure 3.5B, lower part).

Effect of tolerance induction on cytokine production

Furthermore, we investigated whether the increased number of CD4^{+}CD25^{+}Foxp3^{+} T cells also demonstrated a change in the production of cytokines in response to stimulation with HSP60. This may contribute to the decreased plaque size in the mice orally treated with HSP60. Mesenteric lymph node cells, isolated 14 days after the oral treatment with HSP60, were stimulated in vitro in presence or absence of 20 μg/ml of HSP60. Incubation of these lymph node cells with HSP60 resulted in a significant larger production of TGF-β (1.86±0.22 versus 0.93±0.15 ng/ml; $P<0.05$) and IL-10 (19.52±5.51

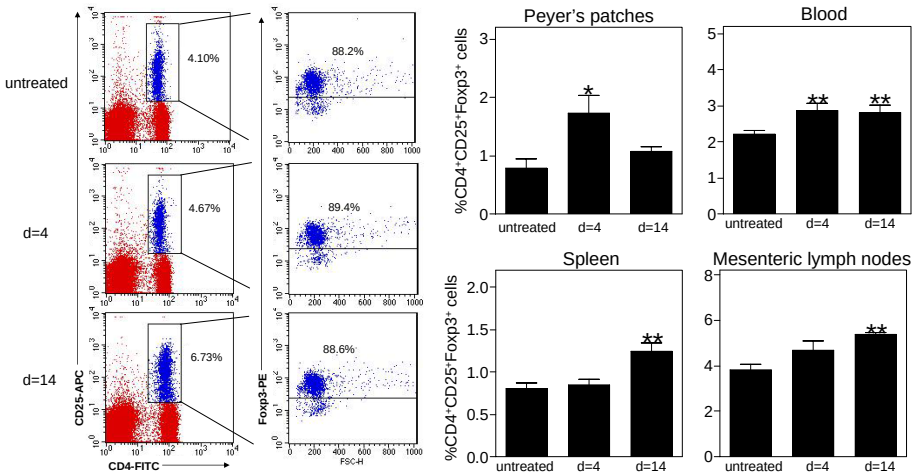

Figure 3.5: Oral tolerance induction to HSP60 leads to an increased amount of $CD4^+CD25^+Foxp3^+$ cells. $LDLr^{-/-}$ mice were fed HSP60 four times and killed 4 and 14 days after oral treatment. As a control, untreated animals were used. The dot plots show representative examples of lymphoid cells isolated from mesenteric lymph nodes stained for CD4 and CD25 (left panel). The right panels show the percentage of $Foxp3^+$ cells within the $CD4^+CD25^+$ population. The graphs represent the amount of $CD4^+CD25^+Foxp3^+$ cells in the Peyer's patches, blood, spleen and mesenteric lymph nodes (mean±SEM). $^*P<0.05$, $^{**}P<0.01$.

versus 6.41±1.72 pg/ml; $P<0.05$) when compared with mesenteric lymph node cells cultured without HSP60 (Figure 3.6A and 3.6B). Furthermore, HSP60-stimulated mesenteric lymph node cells isolated from HSP60-treated mice (14 days after treatment) produced significantly more TGF-β than HSP60-stimulated mesenteric lymph node cells isolated from untreated mice (1.86±0.22 ng/ml versus 0.96±0.22 ng/ml; $P<0.05$; data not shown). In all cases IFN-γ levels were below the detection threshold.

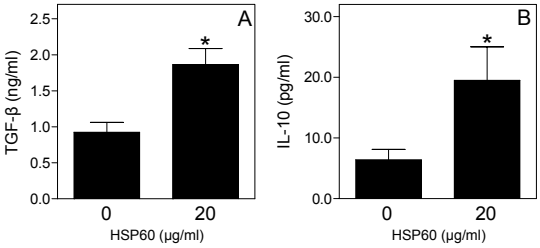

Figure 3.6: Oral tolerance induction to HSP60 induces anti-atherogenic cytokine production by mesenteric lymph node cells. $LDLr^{-/-}$ mice were treated orally with HSP60 four times. 14 days after the treatment, mesenteric lymph nodes were isolated from HSP60-treated mice and the lymphocytes were cultured *in vitro* with or without HSP60 for 48 hours. The production of TGF-β (A) and IL-10 (B) was monitored using ELISA. Data are mean±SEM. $^*P<0.05$.

Regulatory T cell markers in atherosclerotic plaques

After tolerance induction to HSP60 and the induction of atherosclerosis, carotid arteries were dissected and mRNA was isolated. Subsequently, the expression of

different Treg markers (CD25, CTLA-4 and Foxp3) in the atherosclerotic plaques in the carotid arteries was determined. After oral treatment with HSP60 (n=5) and 8 weeks of Western-type diet feeding, the mRNA expression of CD25, CTLA-4 and Foxp3 was significantly upregulated in the atherosclerotic plaque when compared with control mice (n=9). CD25 showed a 4.9-fold increase ($P<0.05$), CTLA-4 a 4.1-fold increase ($P=0.068$) and Foxp3 a 6.4-fold ($P<0.05$) increase (Figure 3.7).

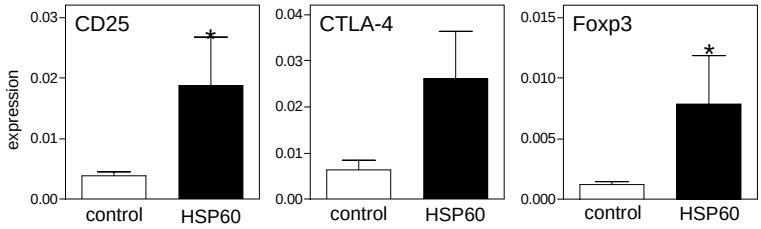

Figure 3.7: Increased expression of Treg markers is observed within lesions of HSP60-treated LDLr$^{-/-}$ mice. To investigate the presence of Tregs within atherosclerotic lesions, mRNA was isolated from carotid arteries of PBS (n=9) and HSP60-treated (n=5) mice and the mRNA expression of CD25, CTLA-4 and Foxp3 was quantitatively determined and expressed relative to 36B4. $^*P<0.05$

Effect of oral tolerance to HSP60 on HSP60-specific antibodies

After oral treatment with HSP60 and the induction of atherosclerosis, HSP60-specific IgG1, IgG2a and IgM levels in serum were determined. No detectable differences in HSP60-specific IgG1, IgG2a and IgM levels were observed (Figure 3.8).

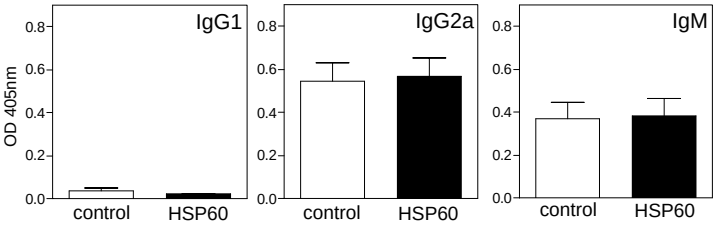

Figure 3.8: Effect of tolerance induction to HSP60 on HSP60-specific antibody-levels. LDLr$^{-/-}$ mice in which atherosclerosis was induced by a combination of Western-type diet feeding and collar placement around both carotid arteries were treated 4 times intragastrically with PBS or HSP60 and serum levels of HSP60-specific IgG1, IgG2a and IgM were measured using a capture enzyme-linked immunosorbent assay. Values are mean OD(405) values±SEM.

Discussion

Previous studies have demonstrated the importance of HSPs in the pathology of atherosclerosis. HSP47 is expressed in fibrous regions of human atheroma and is regulated by growth factors and oxidized lipoproteins.[35] HSP47 is also associated with collagen production.[36] Furthermore, it is known that there is increased HSP70 expression at different sites of atherosclerosis and that oxLDL induces HSP70 expression on human smooth muscle cells[37] and endothelial

cells.[38] However, the HSP with the biggest impact on atherosclerosis is HSP60. Autoantibodies to HSP60 cause endothelial damage[12] and macrophage lysis[39] and are associated with an increase in susceptibility in atherosclerosis. T cells reactive to HSP60 are found to correlate with early atherosclerotic events[40] and are found in atherosclerotic plaques in rabbits[41] and humans.[42] Furthermore, *Chlamydia pneumoniae*, Gram-negative bacteria often linked with atherosclerosis, can induce an immune reaction because of its HSP60 expression, which is highly homologous to human and mouse HSP60. In the current study we show that LDLr$^{-/-}$ mice contain T cells specific for HSP60 and for the small peptide HSP60(253-268), but no T cells with any reactivity against the HSP70(111-125) peptide. HSP60 was derived from *Mycobacterium bovis* bacillus, but due to the high degree of amino acid sequence homology between different species, T cells specific for this HSP60 were found to be cross reactive against self-HSP60[8]. A spleen cell proliferation assay demonstrated a 2-3-fold increase in T cell proliferation in response to 20 μg/ml HSP60 or HSP60(253-268). Immunization of LDLr$^{-/-}$ mice with HSP60 or HSP60(253-268) and a subsequent proliferation assay with 20 μg/ml HSP60 or HSP60(253-268) resulted in a 13- and 9-fold increase in proliferation, respectively, when compared with the non-stimulated splenocytes. Even a lower concentration (5 μg/ml) was sufficient to trigger the splenic T cell population and resulted in a 7-fold increase in proliferation. These data confirm that HSP60 but also the small HSP60-peptide can induce a T cell response in LDLr$^{-/-}$ mice, while the small HSP70-peptide was not effective in these mice

Intervention in the anti-HSP60 autoimmune response could be beneficial for atherosclerosis. Many strategies are used to interrupt autoimmune responses directed towards autoantigens and one of these strategies used in animal models for Th1-mediated autoimmune diseases is mucosal tolerance induction. Mucosal tolerance induction, subdivided in oral and nasal tolerance induction, can lead to a deletion of Th1 and Th2 cells or to an activation of Tregs depending on the administered dose of the antigen. Tregs, induced by low doses of the antigen, are known for the production of TGF-β and IL-10, which both have anti-atherogenic properties. Recently Mallat et al. hypothesized that in atherosclerosis an imbalance exists between pathogenic T cells (Th1 and/or Th2) and Tregs (Tregs) specific for 'altered' self and non-self antigens (e.g. oxidized phospholipids, heat shock proteins).[20] Ait-Oufella et al. showed that Tregs play an important role in controlling the development of atherosclerosis in mice.[19] Recently is was shown that a transfer of *in vitro* generated HSP60-specific Tregs to RAG1$^{-/-}$LDLr$^{-/-}$ mice reduced the development of atherosclerotic lesions.[43] Consequently, mucosal tolerance induction and the subsequent activation of Tregs may be a useful strategy to ameliorate atherosclerosis. Several studies already showed the potency of oral tolerance induction on atherosclerosis. Oral tolerance induction to β2-glycoprotein I,[27] HSP65[28,29] and oxLDL[30] results in the suppression of early atherosclerosis. However, these studies do not give a clear explanation for the observed reduction in atherosclerosis and they do not show whether Tregs are involved in this process.

In our current study we show that oral tolerance induction to HSP60 and HSP60(253-268) attenuates atherosclerosis. A relatively low dose of HSP60 (30 μg, four times) significantly reduced early atherosclerotic lesion formation by

80.7%, reflected in the intima/lumen ratio (68.8% reduction). We now clearly show that an immunogenic peptide present in HSP60 based on the highly conserved sequence 253-268, and capable of inducing a T cell response cross reactive with self-HSP, can also induce tolerance and reduces plaque size by 83.3% and the intima/lumen ratio by 74.3%. The specificity of the response and the involvement of T cells is reflected by the finding that HSP70(111-125), a peptide based on a conserved sequence found in the HSP70 protein of men, rats, and mice, was not effective in reducing atherosclerosis, which may be explained by its inability to induce T cell responses. The experimental setup of our current study is comparable with two previous studies on oral tolerance induction to HSP65/HSP60.[28,29] Both Harats et al.[28] and Maron et al.[29] show a decreased proliferation of splenocytes after oral treatment but no effects on Tregs were described. Maron et al.[29] observed a decreased IFN-γ and an increased IL-10 production by lymphocytes after oral treatment with HSP65. This could indicate an activation of Tr1 cells, a subset of adaptive Tregs, particularly producing IL-10. In our previous study on oral tolerance induction to oxLDL we already showed $CD4^+CD25^+Foxp3^+$ cells to be responsible for the reduction in atherosclerosis. In our current study, we show that T cells specific for HSP60 epitopes are involved in the regulation of atherosclerosis. Low doses (30 μg) of HSP60 and HSP60(253-268) were administered and therefore we investigated the possible activation of Tregs. Four days after the oral HSP60-treatment, the number of $CD4^+CD25^+Foxp3^+$ Tregs was significantly increased in Peyer's patches and blood, as compared to untreated mice. After two weeks, the number of $CD4^+CD25^+Foxp3^+$ T cells was significantly increased in blood, mesenteric lymph nodes and spleen. In the Peyer's patches, the first site of activation, the number of Tregs decreased after two weeks, which may be attributed to the migration of the activated $CD4^+CD25^+Foxp3^+$ T cells to peripheral lymphoid organs and the site of inflammation (atherosclerotic lesions). The Tregs may recognize self-HSP60 known to be upregulated in atherosclerotic lesions. Therefore we also investigated the mRNA-expression of markers for Tregs within the lesions and we observed an increased expression of CD25, CTLA-4 and Foxp3. In addition, oral treatment with HSP60 reduced the proliferative response of splenocytes to HSP60. This dampened response is in line with the studies by Maron et al. and Harats et al.[28,29] Mesenteric lymph node cells of HSP60-treated mice produced an increased level of TGF-β (2.0-fold) and IL-10 (3.1-fold) after *in vitro* re-stimulation with HSP60.

Natural Tregs, which are $CD4^+CD25^+Foxp3^+$ T cells can display their specific immunosuppressive effects via TGF-β on their surface, which binds to TβRII expressed on T cells specific for the same antigen. TGF-β-TβRII interaction leads to the activation of a Smad-dependent pathway, resulting in a blockade of IL-2 production and a reduced proliferation of HSP60-specific T cells. CTLA-4 is also important in the cell-cell interaction between Tregs and other T cells. It is however more likely that adaptive Tregs (Th3 and Tr1 cells) are involved in oral tolerance induction, because the natural Tregs are thymus-derived and can not be activated in the periphery. Th3 cells, which can be activated in the periphery, are known for the production of anti-inflammatory TGF-β and upon activation they may express Foxp3. Tr1 cells, which can also be activated in the periphery, produce

particularly anti-inflammatory IL-10 but whether these Tregs express Foxp3 is still not clear. It is also known that Th3 cells are especially activated via oral tolerance induction and Tr1 cells via nasal tolerance induction. Therefore we assume that in this study, oral tolerance induction led to an increase in Foxp3-expressing Th3 cells, producing excessive amounts of TGF-β but also IL-10. This IL-10 may however also be produced by induced Tr1 cells, but these cells do not contribute to the increase in Foxp3$^+$ Tregs.
In conclusion we describe that LDLr$^{-/-}$ mice can be tolerized to HSP60 and a HSP60 peptide (HSP60(253-268)) which results in an attenuation of early atherosclerotic lesions. The mechanism underlying this effect can be attributed to the induction of CD4$^+$CD25$^+$Foxp3$^+$ Tregs by oral HSP60 administration. These Tregs could produce TGF-β and IL-10 upon recognition of self-HSP60 known to be upregulated in atherosclerotic lesions. In this way they can down-regulate the inflammatory response locally.[8] Altogether, these beneficial results of oral tolerance induction to HSP60 and HSP60(253-268) on atherosclerosis may provide new therapeutic approaches for the treatment of atherosclerosis.

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Chapter 4

Immunotherapy of atherosclerosis using dendritic cells

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-Submitted for publication-

Abstract
Modification of lipoproteins plays an important role in the development of atherosclerosis. OxLDL has a number of pro-inflammatory effects, whereas immunization with various forms of oxLDL is able to reduce atherosclerosis. The uptake of modified LDL by dendritic cells (DCs) and the presentation of epitopes thereof may form an important step in the immunomodulatory effects of LDL. In this study we transferred oxLDL-pulsed DCs to $LDLr^{-/-}$ mice and examined the effects on atherosclerosis. Bone marrow derived DCs were cultured for 10 days in the presence of GM-CSF. Immature DCs were matured by LPS and pulsed with cupper oxidized LDL (Cu-oxLDL). These cells were transferred to $LDLr^{-/-}$ mice every other day before induction of atherosclerosis by Western-type diet feeding. Transfer of oxLDL-pulsed DCs resulted in a 92% and 87% reduction in carotid artery lesion size compared to the PBS-treated mice or mice treated with mature DCs (mDCs), respectively ($P<0.001$). Lesion size in the aortic valves was not affected but we observed at both sites an increase in plaque stability. The reduction in atherosclerosis was accompanied by a 3.8-fold increase ($P<0.05$) in Cu-oxLDL specific IgG levels whereas the levels of MDA-LDL-specific IgG and IgM were not significantly affected. In addition, we also showed that the serum of mice treated with oxLDL-pulsed DCs reduced the formation of foam cells as compared to serum from PBS-treated mice or mice treated with mDCs. We conclude that oxLDL-pulsed DCs induce an enhanced production of anti-oxLDL IgG levels which leads to a reduction in atherosclerosis by modulating the immunostimulatory effects of oxLDL. These data indicate that vaccination with oxLDL-pulsed DCs provides a novel and powerful strategy in the treatment of atherosclerosis.

Introduction

Atherosclerosis is a slowly progressing disease that develops at sites of lipid accumulation in large and medium sized arteries, which can lead to infarction of the heart or the brain. Over the past several years, accumulating data has identified a key role for inflammation in atherosclerosis and both innate and adaptive immune responses are involved.[1–5] Several antigens have been implicated in the initiation of immune responses during atherosclerosis including exogenous infectious pathogens such as *Chlamydia pneumoniae*, and cytomegalovirus but also endogenous proteins such as heat-shock proteins and β2-glycoprotein-Ib.[6–8] The most intensively studied endogenous antigen is oxidized low-density lipoprotein (oxLDL). Oxidation of lipoproteins in the arterial intima, followed by their uptake by macrophages and subsequent foam cell formation, plays an important role in the development of atherosclerosis. Also, peroxidization of polyunsaturated fatty acids in phospholipids and cholesteryl esters generates highly reactive breakdown compounds such as malondialdehyde (MDA) and 4-hydroxynonenal that cause cell damage and local inflammation.[9–11] In addition, oxidation of LDL results in many structural modifications of apoB-100 and thus the formation of many neo-epitopes, which renders the modified LDL immunogenic and leads to both a cellular and humoral response. Since the different epitopes of oxLDL induce atherogenic immune responses, it is attractive to modulate the immune response towards oxLDL. Also, a number of studies show that immunization against oxLDL reduces atherosclerosis in several animal models.[12–14]

OxLDL also plays a role in the maturation of dendritic cells (DCs). DCs are the most potent antigen-presenting cells of the immune system.[15–17] In the periphery they act as sentinels for the innate immune system whereas in the lymph nodes they are the key messengers in adaptive immunity. Immature DCs differentiate from bone marrow progenitors or circulating blood monocytes. They reside in the blood stream or peripheral tissues where they survey incoming pathogens. An interaction with pathogens induces maturation during which DCs generate MHC-peptide complexes and upregulate the expression of costimulatory molecules such as CD40, CD80 and CD86. These changes render the DCs fully competent to activate T cells. Several studies showed that oxLDL induces several changes, characteristic for DC maturation, including a higher expression of costimulatory molecules and the increased ability to stimulate T cells.[18,19]

Due to their potent capacity to stimulate T cells, DCs are being investigated in vaccine and therapy approaches.[20,21] We wanted to assess the use of oxLDL-pulsed DCs as an immunotherapy for atherosclerosis. DCs obtained from the bone-marrow can be pulsed *ex vivo* by inducing maturation in the presence of oxLDL. Such cultivation of DCs will mimic the *in vivo* processing of oxLDL by DCs. In this study we show that repeatedly injection of oxLDL-pulsed DCs induced an enormous reduction in lesion size and increased the production of oxLDL-specific antibodies (Ab). The high titers inhibit foam cell formation and thus prevent the negative effects of oxLDL on the arterial wall. In conclusion, these data indicate that vaccination with oxLDL-pulsed DCs provides a novel and powerful strategy in the battle against atherosclerosis.

Methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr$^{-/-}$ mice and UBC-GFP mice were from Jackson Laboratories on a C57Bl6 background and bred in-house. Male C57BL/6J mice were from Charles River Laboratories. Mice were kept under standard laboratory conditions and were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Mice were 10-12 weeks old at the start of the experiment. Diet and water were administered *ad libitum*.

Media and reagents

The cell culture medium used for dendritic cells was IMDM (Cambrex, Belgium) supplemented with 8 % FBS (PAA, Germany), 100 U/ml streptomycin/penicillin (PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μM β-mercaptoethanol. LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave et al.[22] The isolated LDL was dialyzed against phosphate buffered saline (PBS) with 10 M EDTA (pH 7.4) for 24 hours at 4°C and oxidized by exposure to 10 μM $CuSO_4$ at 37°C for 20 hours as previously described.[23]

Generation and injection of BM-DCs

For each injection time-point, bone marrow cells were isolated from the tibia and femora of 3 C57BL/6 mice. Cells were immediately pooled and cultured during 10 days in complete IMDM in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF). After 10 days of culture, immature DCs were activated with 1 μg/ml of lipopolysaccharide (LPS from Salmonella typhosa, Sigma Aldrich, Zwijndrecht, The Netherlands) during 24 hours. Simultaneously, DCs were pulsed with or without 7.5 μg/ml of cupper-oxidized LDL. Purity and functionality of the DCs were assessed using flow cytometry. CD11c was used as a specific DC marker (purity $>$ 90%). Functionality was determined by the expression of several costimulatory molecules (CD40, CD80, CD86) and the expression of MHC-II and CD1d. Mice were injected intravenously (8, 6 and 3 days prior to atherosclerosis induction by Western-type diet feeding) with 200 μl of PBS (n = 8) or $1.5{\cdot}10^6$ DCs (oxLDL-pulsed or unpulsed mDCs) in 200 μl PBS (n = 11). DCs from the UBC-GFP mice were identically isolated en cultured.

Cytospin

300 μl of cell suspensions were centrifuged for 5 min at 500 rpm using the Thermo Shandon Cytospin 4. After centrifugation, slides were fixed for 30 minutes using zinc formal Fixx (Shandon, Pittsburgh, USA). Lipid loading in the DCs was visualised using Oil-Red-O staining.

Induction of atherosclerosis

Male LDLr$^{-/-}$ mice were injected 3 times intravenously (1 injection every other day) with $1.5 \cdot 10^6$ DCs or saline prior to Western-type diet feeding. After 3 weeks of diet, atherosclerosis was induced by bilateral perivascular collar placement (2 mm long, diameter 0.3 mm) around both carotid arteries and continuous Western-type diet feeding.[24] During the experiment, plasma samples were obtained by tail vein bleeding. 7 weeks after collar placement, mice were sacrificed and tissues were harvested after *in situ* perfusion using PBS and subsequent perfusion using formalin. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use.

Histological analysis and morphometry

Transverse 5 μm cryosections were prepared in a proximal direction from the carotid bifurcation. Cryosections were stained with hematoxylin (Sigma Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica, Germany) or with hematoxylin and Oil-Red-O. Hematoxylin-eosin stained sections of right carotid artery and Oil-Red-O stained sections of heart valves were used for morphometric analysis of atherosclerotic lesions as described previously.[24] Corresponding sections were stained immunohistochemically with antibodies against a macrophage specific antigen, (MOMA-2, polyclonal rat IgG2b, Research Diagnostics Inc, NJ) or were stained for collagen fibers using the Masson's Trichrome method (Sigma Aldrich, Zwijndrecht, The Netherlands). The site of maximal stenosis was used for assessment. The images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, Cambridge, UK).

Flow Cytometry (FACS analysis)

Antibodies used for staining were purchased from eBiosience, Belgium. Staining was done in PBS in the presence of 1 % mouse serum. FACS analysis was performed on the FACSCalibur (Becton Dickinson, Mountain View, CA). Data were analyzed using Cell Quest software.

Cholesterol assay

Blood was collected at several time points during the experiment by tail vein bleeding. Plasma was obtained after centrifugation and total plasma cholesterol levels were measured using enzymatic procedures and a spectrophotometer (Roche Diagnostics, The Netherlands). Precipath was used as an internal standard (Boehringer, Mannheim, Germany). The cholesterol distribution over the different lipoproteins was analyzed by fractionation using a Superose 6 column (3.2 x 30 mm, Smart-System, Pharmacia).

ELISA for IgM and IgG antibodies against LDL, MDA-LDL and Cu-oxLDL

Antibodies against LDL, MDA-LDL and Cu-oxLDL were determined according to Damoiseaux et al.[25] MaxiSorp 96 well plates (Nunc, Roskilde, Denmark) were

coated overnight with 100 μg native LDL or oxLDL in 100 μl PBS at 4°C. Plates were washed 5 times with 0.01M Tris, 0.15 M NaCl and 0.05% Tween20 (pH 8.0). Mouse serum was added in duplicate at a 1:50 dilution in incubation buffer (0.1 M Tris, 0.3 M NaCl and 0.05% Tween20 (pH 8.0) overnight at 4°C. After washing, plates were incubated with either alkaline phosphatase-labelled anti-mouse IgM or IgG (Jackson Immuno-Research, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. After washing, substrate (1 mg/ml disodium p-nitrophenyl phosphate, Sigma, The Netherlands) was added. After 2 hours at room temperature, absorbance was read at 405 nm.

ELISA for detecting subclasses specific Ab

IgG1 and IgG2A levels were detected using the Mouse MonoAb ID kit (Zymed Laboratories Inc., South San Fransisco, USA) according to manufacturers' instructions. Briefly, high binding plates were coated overnight with 50 μl Cu-oxLDL (5 μg/ml). Serum samples were 1:1 diluted in PBS. IgG1 and IgG2A levels were detected using a spectrophotometer at 405 nm.

Foam cell formation

To obtain macrophages, bone-marrow cells from C57BL/6 mice were resuspended in complete RPMI supplemented with 20% FCS and 30% of L929-conditioned medium (source for monocyte-colony-stimulating factor, M-CSF). After 7 days of culture, macrophages were seeded in Lab-Tek chamber slides (BD Falcon, The Netherlands) ($0.8 \cdot 10^6$ cells/well) in cRPMI $^+$ 10% FCS. After 4 hours of incubation, cells were washed and resuspended in cRPMI without FCS. After 4 hours, a mixture of oxLDL (10 μg/ml) plus an equal volume of mouse serum (2 fold diluted, serum from 2 mice were pooled) was added. This mixture of oxLDL and mouse serum was made 30 minutes before addition to the cells. After overnight culture, cells were fixed using zinc formal Fixx, stained for lipids using Oil-Red-O and counter stained with hematoxylin. The amount of Oil-Red-O staining was corrected for the number of cells as indicated by the hematoxylin staining. 8 random fields per condition were analysed using the Leica DM-RE microscope and LeicaQwin software.

Statistical analysis

Values are expressed as mean $\pm$ SEM. Differences between different treated groups were assessed with parametric or non-parametric ANOVA followed by a t-test using the Instat3 software. To measure the effects on cholesterol, the repeated measures ANOVA was used. Probability values of $P<$ 0.05 were considered significant.

Results

Effect of oxLDL on dendritic cells

Firstly we investigated whether DCs did engulf oxLDL and whether the uptake of lipoproteins by DCs did affect the maturation of DCs. During maturation, DCs

upregulate the expression of costimulatory molecules and the maturation level of DCs can thus be followed by flow cytometry of several typical markers (CD40, CD80, CD86, MHCII, and CD1d). Increasing concentrations of oxLDL (up to 20 μg/ml) were added to immature DCs. After 24 hours of incubation we stained the intracellular lipid accumulation in the DCs and found that increasing oxLDL concentrations induced an increasing lipid accumulation. However, oxLDL levels exceeding 7.5 μg/ml induced a concentration dependant increase in cell death and a lowering of the expression of the DC-specific markers (data not shown). Therefore we have chosen a concentration of 7.5 μg/ml which induced optimal lipid loading but no cell death.
We treated immature DCs (Figure 4.1A) with the TLR4 agonist, LPS (1 μg/ml), in the absence or presence of oxLDL (7.5 μg/ml). After 24 hours of incubation, DCs were collected and stained with Oil-Red-O and analyzed for surface markers by flow cytometry. The addition of oxLDL to both imDCs and mDCs resulted in lipid accumulation in more than 95% of the DCs (Figure 4.1B and 4.1C respectively). Treatment with LPS resulted in a typical mature DC cell surface phenotype showing high expression of CD11c (a specific marker for mouse DCs), MHCII, CD1d and the costimulatory molecules CD40, CD80, CD86. The addition of oxLDL during maturation had no effect on the expression level of these molecules and thus did not affect the maturation state of the DCs (Figure 4.1D).

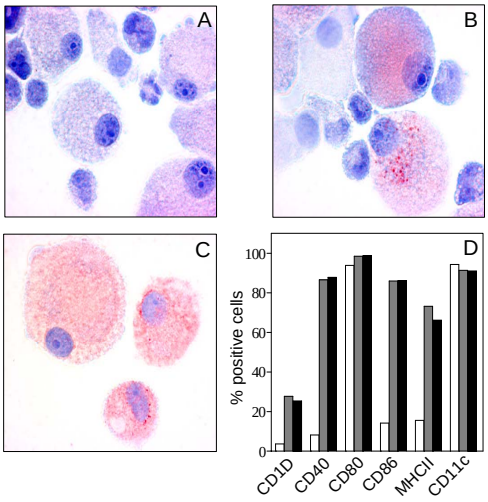

Figure 4.1: The effect of oxLDL on dendritic cells. Immature DCs were treated with LPS (1 μg/ml) in the absence or presence of oxLDL (7.5 μg/ml). After 24 hours, DCs were collected. Oil-Red-O staining of DCs: immature DCs in the absence of oxLDL (A), in the presence of oxLDL (B) and mature DCs in the presence of oxLDL (C). Expression levels of surface markers analysed by FACS on immature DCS (white bars), mature DCs (grey bars) and mature oxLDL-pulsed DCs (black bars).

Homing of dendritic cells.

To determine the fate of DCs *in vivo*, we cultured DCs from UBC-GFP mice, which have a high and constitutive expression of GFP and injected $1.5{\cdot}10^6$ DCs into LDLr$^{-/-}$ mice. We injected both mature unpulsed and mature oxLDL-pulsed

GFP-DCs and 72 hours after injection, mice were sacrificed and GFP expression was determined in different tissues using flow cytometry. While there were almost no GFP^+ DCs present in the blood, DCs did migrate towards the lung and liver, and towards organs involved in immune responses such as spleen, mediastinal and inguinal lymph nodes (Figure 4.2). Mature unpulsed and mature oxLDL-pulsed DCs displayed an identical distribution pattern.

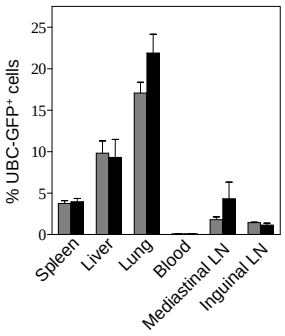

Figure 4.2: Homing of GFP^+-DCs. $1.5{\cdot}10^6$ mature GFP^+-DCs (grey bars, n=4) and mature oxLDL-pulsed GFP^+-DCs (black bars, n=4) were injected i.v. into $LDLr^{-/-}$ mice. Mice were sacrificed after 72 hours. GFP^+ cells as percentage of the total number of leukocytes in the different organs are shown.

Effect of vaccination using oxLDL-pulsed DCs on atherosclerosis

To evaluate the effect of vaccination using oxLDL-pulsed DCs on de novo atherosclerosis, $LDLr^{-/-}$ were injected intravenously 3 times (day -8, -6 and -3) with either PBS (n = 8), mDCs (n = 11) or oxLDL-pulsed DCs (n = 11). On day 0, mice were put on a Western-type diet. Three weeks thereafter, atherosclerosis was induced by perivascular collar placement around the carotid arteries and subsequent Western-type diet feeding. Seven weeks after collar placement, mice were sacrificed and plaque formation in the right carotid artery and the aortic root were analyzed using the hematoxylin/eosin staining and hematoxylin/Oil-Red-O staining, respectively. Figure 4.3A-C show representative examples of hematoxylin/eosin staining in the carotid arteries. Injection of oxLDL-pulsed DCs resulted in a significant 92% ($48578\pm9231\ \mu m^2$ vs $4024\pm504\ \mu m^2$; $P<0.001$) or 87% ($31919.8\pm7914\ \mu m^2$ vs $4024\pm504\ \mu m^2$; $P<0.001$) reduction in plaque size in the carotid arteries compared to the PBS-treated or mDC-treated groups, respectively (Figure 4.3D). Also a concomitant reduction in intima/lumen ratio of 87% (0.560 ± 0.097 vs 0.071 ± 0.009; $P<0.001$) or 85% (0.461 ± 0.089 vs 0.071 ± 0.009; $P<0.001$) was observed (Figure 4.3E). In contrast, we did not observe significant effects of treatment with dendritic cells on plaque size in the aortic roots (PBS: $199411\pm17408\ \mu m^2$; mDCs: $293181\pm36193\ \mu m^2$; mDCs plus oxLDL: $224983\pm48546\ \mu m^2$) (Figure 4.3F).

Plaque morphology was assessed using the MOMA-2 staining for detecting macrophages and the Masson's Trichrome method was used for visualizing collagen fibers. In the carotid arteries we observed non-significant decreases in the MOMA-2/intima ratio (Figure 4.4A). The macrophage content was also reduced in the aortic root, where we saw a significant 2.5-fold reduction compared to the PBS-treated group ($P<0.05$) and a non-significant 1.9-fold reduction in MOMA-2/intima ration compared to the mDC-treated group (Figure 4.1D). The collagen content of the intima was increased in both the carotid arteries (Figure 4.4B; $P<0.05$) and the aortic root (Figure 4.4E). Additionally, we calculated the MOMA-2/collagen ratio as an indicator for plaque stability.

There was a significant 4.3-fold ($P<0.05$) and 4.1-fold ($P<0.05$) reduction in MOMA-2/collagen ratio in the group treated with oxLDL-pulsed DCs compared to the group treated with PBS and a significant 3.9-fold and 2.8-fold reduction compared to the group treated with mDCs in both the carotid arteries and aortic root, respectively (Figure 4.4C and 4.4F). This reduced MOMA-2/collagen ratio indicated a more stable plaque phenotype in the mice treated with oxLDL-pulsed DCs.

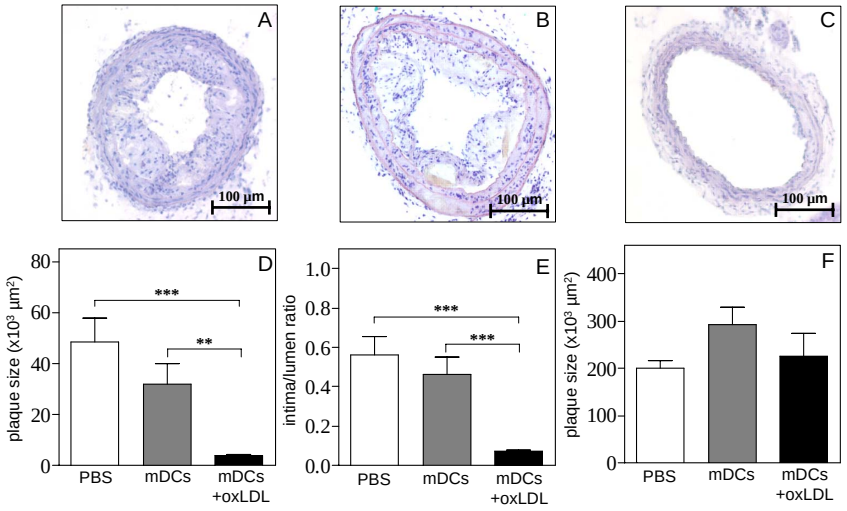

Figure 4.3: Effect of DC vaccination on atherosclerosis. Representative sections of lesions in the carotid arteries of PBS-treated (A), mDC-treated (B) and oxLDL-pulsed DC-treated (C) mice. Lesion size (D) and intima/lumen ratio (E) in the carotid arteries were determined. Plaque size in the aortic root was quantified after hematoxylin/Oil-Red-O staining (F). (n=7-11 per group; $^{**}P<0.01$, $^{***}P<0.001$)

Effect of vaccination on total cholesterol levels

Both body weight and total cholesterol levels were measured at different time points during the experiment. There were no significant differences in body weight between the different groups throughout the entire experiment. As shown in figure 4.5A, cholesterol levels of the PBS and mDC-treated groups kept on increasing in time, whereas the cholesterol levels in the group treated with oxLDL-pulsed DCs levelled off after 7 weeks. The repeated measurements ANOVA did not result in significant differences over time. It remains however interesting that at sacrifice, mice treated with oxLDL pulsed DCs showed significant 30% ($P< 0.05$) and 27% ($P< 0.01$) lower cholesterol levels compared to mice treated with PBS and mDCs, respectively. The reduction did not result from the lowering of one particular class of lipoproteins (VLDL, LDL or HDL) as depicted in the lipoprotein profile (Figure 4.5B).

Humoral response after vaccination

Plasma samples from each mouse were obtained after sacrifice and IgG, IgM and subclass specific antibodies were determined. The T cell-dependent IgG antibodies against Cu-oxLDL were significantly higher in mice vaccinated with

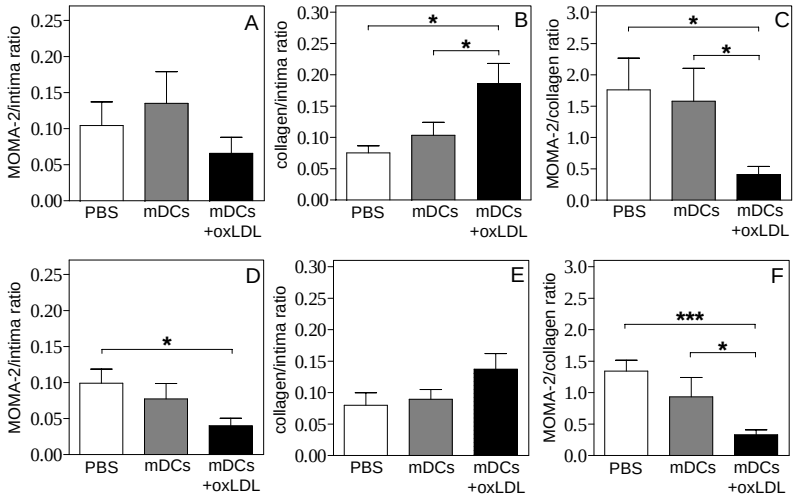

Figure 4.4: Plaque morphology. LDLr$^{-/-}$ mice were treated with PBS, mDCs or mature oxLDL-pulsed DCs. Subsequently mice were put on a Western-type diet and 10 weeks later plaque composition was assessed in the carotid artery (A-C) and in the aortic root (D-F). The amount of macrophages was determined as the MOMA-2 positive area per intima area (A and D), the amount of collagen was determined by quantifying the blue collagen staining of the Masson's Trichrome stain within the plaque (B and E). The plaque stability was quantified as MOMA-2/collagen ratio (C and F) (n=7-10 per group; $^{*}P<0.05$), $^{***}P<0.001$

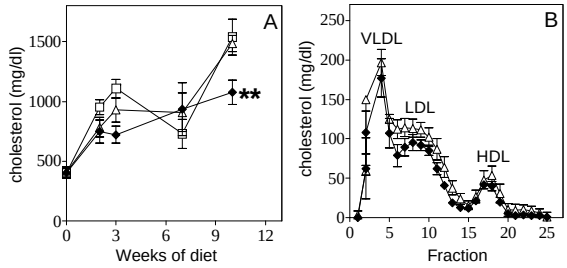

Figure 4.5: Effect on total plasma cholesterol. Cholesterol levels were determined in plasma of the different mouse groups (PBS (open squares), mDCs (open triangles) and oxLDL-pulsed mDCs (closed diamond)) at the indicated time points (A). Serum was separated on a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles (B). (n=8-11 per group; $^{**}P<0.01$).

oxLDL-pulsed DCs compared to the mice treated with PBS or mDCs (Figure 4.6A) ($P< 0.001$ and $P< 0.01$, respectively). Interestingly no significant effect was found on the IgG levels against MDA-LDL which indicates a highly specific response against Cu-oxLDL (Figure 4.6B). No differences in titers of IgM against MDA-LDL or Cu-oxLDL were detected between the various groups (Figure 4.6C and D, respectively). We also analyzed whether IgG1 or IgG2a mainly contributed to the rise in anti-oxLDL IgG levels and we observed significantly higher levels of both IgG1 and IgG2A in the mice treated with oxLDL-pulsed DCs compared to the mDC-treated group indicating that the production of anti-oxLDL antibodies was not restricted to a Th1 or Th2 response (data not shown).

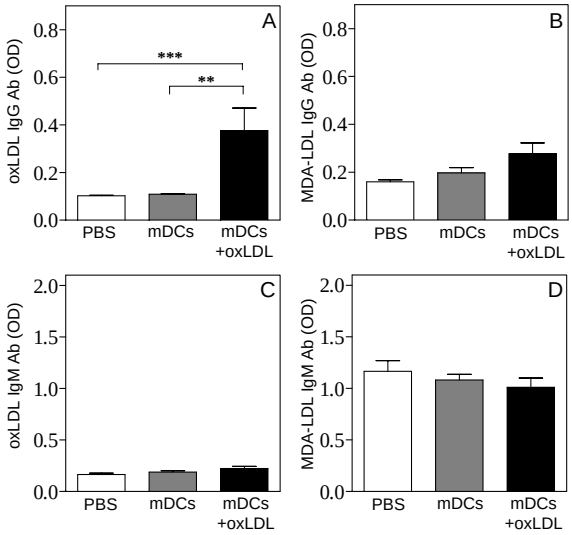

Figure 4.6: Humoral response against oxLDL and MDA-LDL. At the end of the experiment, IgG and IgM titers were determined in serum of mice treated with PBS, mDCs and mature oxLDL-pulsed DCs using an ELISA. IgG titers against oxLDL and MDA-LDL are presented in A and B, respectively. Figures C and D show IgM levels against oxLDL and MDA-LDL, respectively. (n=8-11 per group; $^{*}P<0.05$).

Cellular response to vaccination strategy

To evaluate the effect of DC treatment on a number of different subsets of leukocytes, blood was collected before injection of DCs, after each injection, before collar placement and at sacrifice and FACS analysis was performed on leukocytes. We observed no significant differences in number of blood $CD8^{+}$ or $CD4^{+}CD25^{+}$ cells at any time point during the experiment. We also performed FACS analysis on spleen, liver and mediastinal lymph nodes after sacrifice. There were no differences in number of T cells, regulatory T cells, $CD11c^{+}$ or $CD1d^{+}$ cells (data not shown).

Inhibition of foam cell formation

Macrophages were obtained by culturing bone marrow derived cells from C57Bl6 mice in L929-conditioned medium (source of M-CSF). The phenotype of the macrophages was tested using FACS analysis. Cells were >90% positive for F4/80 and CD11b and <10% positive for CD11c and thus considered to be macrophages (Figure 4.7A-C). Foam cell induction in these macrophages by incubation with oxLDL was hampered by serum from animals treated with oxLDL-pulsed DC (Figure 4.7D). Lipid loading in the foam cells was 3.8 ($P<0.01$) and 2.5 ($P<0.001$) times lower using serum from mice treated with oxLDL-pulsed DC as compared to serum from PBS or mDC-treated mice, respectively.

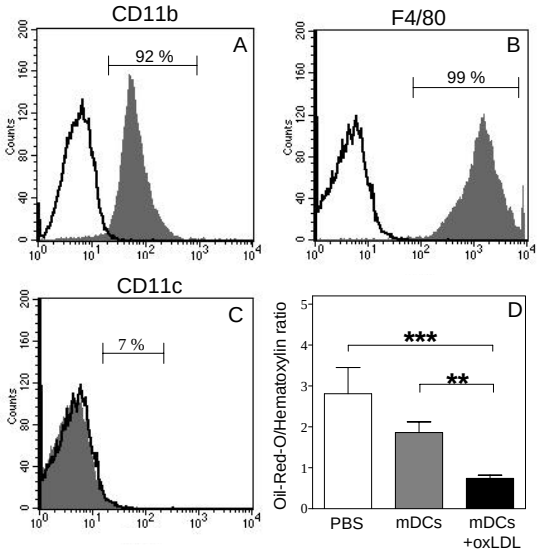

Figure 4.7: Inhibition of foam cell formation. Bone-marrow derived macrophages were analysed by FACS analysis. The fluorescence intensity of respectively CD11b, F4/80 and CD11c (grey curves) are depicted in A, B and C, respectively. The white curves represent the isotype control. The amount of Oil-Red-O staining of macrophages was corrected for the number of cells as indicated by the hematoxylin staining. The addition of mouse serum from mice treated with oxLDL-pulsed DCs (black bars) inhibits the uptake of oxLDL by macrophages as compared to the serum from mice treated with PBS (white bars) and mDCs (grey bars) (D). (n=3, 8 fields per condition) $^{**}P<0.01$; $^{***}P<0.001$.

Discussion

Oxidation of lipoproteins and oxidative processes play an important role in the initiation and progression of atherosclerosis. In this study we show that vaccination using oxLDL-pulsed DCs is a successful strategy in reducing arterial atherosclerotic lesion formation in $LDLr^{-/-}$ mice. In healthy vascular tissue low numbers of DC reside within the intima, immediately beneath the endothelium and in the adventitia along the external elastic lamina.[26,27] In atherosclerosis-prone regions of healthy carotid arteries, the DCs accumulate and form clusters at sites subjected to major haemodynamic stress. This accumulation of DCs at sites prone to develop atherosclerosis has already been established in children from 8 weeks to 10 years old.[26,27] So, vascular DCs become already activated in very early stages of atherosclerosis and induce a primary immune response against the antigens present in the adventitia such as oxLDL. In the advanced atherosclerotic plaques the number of DCs increases by invasion of DCs from the adventitia and from blood. After antigen uptake within the lesions, activated DCs form clusters with T and NKT cells in the shoulder regions of the plaques and may thus play an important role in plaque destabilization.[28,29] It has been suggested that the migratory routes of vascular DCs are similar to those in other peripheral tissues. After uptake of antigen, vascular DCs tend to migrate towards regional lymph nodes where they activate T cells. Importantly, plaque progression was linked to a reduced emigration of monocyte-derived DC-like

cells from developing lesions.[30] In accordance, histopathological studies on human arterial tissues demonstrated that some DCs migrate towards the lymph nodes while other DCs stay within the intima and interact with T cells.[31] Also, hyperlipidemia suppressed the migration of skin DCs.[32] Taken together, these studies emphasize the important immunoregulatory role of vascular DCs and that the impaired migration of DCs may play an important role in the development of atherosclerosis.

We therefore treated atherosclerosis-prone mice with DCs that were loaded *ex-vivo* with modified LDL to prime the immune response against modified LDL, which is one of the main pro-inflammatory antigens in atherosclerosis. At present we show that the use of oxLDL-pulsed DCs resulted in a 92% or 87% reduction in lesion seize in the carotid arteries compared to the PBS or the mDC-treated control groups, respectively. *Ex-vivo* generated and antigen-loaded DCs have been used in vaccination protocols in many animal models[33,34] and have been used to improve immunity in cancer[35] and HIV-infected patients.[36,37] Since the initial study of Palinski[38], a number of studies have shown the effectiveness of immunization with oxLDL or apoB100 peptides as an immunotherapy for atherosclerosis in experimental animal models leading to a reduction in atherosclerosis ranging from 40 to 70% in mice and rabbits.[12–14,39] The use of oxLDL loaded dendritic cells provided us with a simple but very sufficient method of manipulating the immune system and DC-therapy does not require the use of adjuvants. Also, DCs present a broad spectrum of epitopes after internalizing and processing the Ag. Therefore DCs are capable of activating a wide range of Ag-specific T cells and immune escape is minimized. Additionally, we showed that oxLDL is easily engulfed by immature DCs and that the presence of oxLDL during maturation also resulted in the uptake of lipids as shown by the Oil-Red-O staining. By injecting GFP^+ DCs we showed that after 3 days both mature unpulsed and mature oxLDL-pulsed DCs homed in an identical manner to various organs including lymph nodes and spleen, where they can interact with other immune cells and thus induce immune responses, as observed by others.[40]

The observed reduction in lesion size in the carotid arteries was accompanied by a decrease in macrophage numbers and an increase in collagen fibers, leading to a significantly more stable phenotype in the oxLDL-pulsed DC group as compared to both the PBS-treated and mDC-treated group. In contrast, we did not observe a significant reduction in lesion size in the aortic valve leaflets. This discrepancy is similar to that observed by Fredrikson et al. who showed that immunization with apoB-100 induced a 60% reduction in plaque size in the descending aorta but did not affect the size of the subvalvular plaques.[13] On the other hand, we did observe a significant reduction in the number of macrophages in the aortic sinus, indicating a reduced inflammatory status of the lesions in the valve area and we did observe an increase in collagen fibers. Taken together, these data indicated a more stable phenotype in the aortic root and confirmed the effect of oxLDL-pulsed DCs on the composition of the plaque in the carotid arteries.

During the experiment, cholesterol levels were evaluated. Interestingly, after DC vaccination and 10 weeks of diet, a significant reduction in total cholesterol levels in mice treated with oxLDL-pulsed DCs was observed. The lowering of total cholesterol observed was not due to a change in the lipoprotein profile as

shown by SMART analysis. Although this decrease of cholesterol cannot explain the enormous decrease in atherosclerotic lesion formation, since the overall cholesterol load during the experiment was comparable between the two groups, it may form an additional interesting long-term effect of treatment with oxLDL pulsed DCs. The reduction in cholesterol levels observed is in accordance with Freigang et al. who showed a significant reduction in total plasma cholesterol in MDA-LDL immunized LDLr$^{-/-}$ mice after 15 weeks of diet.[14]

The lowering of cholesterol was accompanied by an increase of oxLDL-specific IgG antibodies. We did not detect differences in IgM levels when comparing the three groups. However, we did observe an increase in T cell-dependent IgG Ab towards Cu-oxLDL and not towards MDA-LDL in the group treated with oxLDL-pulsed DCs. This indicates that the treatment with oxLDL-pulsed DCs induced an activation of Cu-oxLDL-specific T cells. In addition, we also showed that the serum of mice treated with oxLDL-pulsed DCs reduced the formation of foam cells as compared to serum from PBS or mDC-treated mice. These mice have higher titers of oxLDL-specific IgG Ab and we argue that the oxLDL-specific IgG can inhibit foam cell formation via complex formation with oxLDL. This is in agreement with the findings of the group of Witztum who showed that monoclonal IgG Fab Ab directed to oxLDL blocked foam cell formation in macrophages.[41] Also, Caligiuri et al. showed that serum from ApoE$^{-/-}$ mice, immunized with phosphorylcholine reduced the uptake of oxLDL by macrophages. In addition, both Zhou et al. and Freigang et al. demonstrated an inverse correlation between lesion size and anti-MDA-LDL IgG levels in mice immunized with MDA-LDL.[14,42] These data suggest that the presence of oxLDL-immune complexes play an atheroprotective role.

Taken together, this strategy of DC vaccination provides a highly efficient route to modulate the immune responses to oxLDL during atherosclerosis and is a new efficient vaccination strategy that is practically feasible.

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Chapter 5

The effect of NKT cell activation on atherosclerosis depends on apoE and lipid loading

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Abstract

It has been shown that NKT cell activation via the administration of α-GalCer accelerates atherosclerosis in apoE$^{-/-}$ mice. ApoE is however an important mediator in the presentation of exogenous lipids via CD1 molecules to NKT cells which may complicate conclusions on the role of NKT cell activation in atherosclerosis. Treatment of LDLr$^{-/-}$ mice with α-GalCer during Western-type diet feeding is therefore of interest to investigate the role of NKT cells in atherosclerosis. Atherosclerosis was induced by the combination of Western-type diet feeding and collar placement around the carotid arteries in both LDLr$^{-/-}$ and apoE$^{-/-}$ mice. Subsequently, the mice were treated twice a week for 7 weeks with α-GalCer. This resulted in a 84% reduction in plaque size in LDLr$^{-/-}$ mice ($P<0.05$), while no effect was observed in apoE$^{-/-}$ mice. *In vitro* incubation of splenocytes with α-GalCer showed that LDLr$^{-/-}$ splenocytes proliferated strongly, while apoE$^{-/-}$ splenocytes show only a minor proliferative response. This is reflected in a larger increase in production of cytokines and especially IL-10 after *in vitro* stimulation of LDLr$^{-/-}$ splenocytes with α-GalCer compared with apoE$^{-/-}$ splenocytes. Additionally, feeding a Western-type diet for 1.5 weeks induced a strong increase in the number of NKT cells within the liver and spleen of LDLr$^{-/-}$ mice. This increase was slower and less prominent in apoE$^{-/-}$ mice which only showed an increased level of NKT cells in the liver after 4.5 weeks of diet. To conclude, administration of α-GalCer to LDLr$^{-/-}$ mice in combination with Western-type diet feeding reduced plaque formation, but this effect was not seen in apoE$^{-/-}$ mice. This may be explained by the decreased presentation of lipids on CD1 molecules due to the lack of apoE. In this study we proved for the first time that NKT cells may also act in an atheroprotective manner.

Introduction

Atherosclerosis is a chronic inflammatory disease of the vasculature in which both the innate and the adaptive immune system play an important role. T cells, B cells, monocytes and dendritic cells (DCs) are detected in atherosclerotic plaques of mice and humans.[1,2] The inflammatory response in atherosclerosis is mainly driven by Th1 cells, producing pro-atherogenic cytokines such as IL-12 and IFN-γ.[3–5] The counteracting Th2 cells produce anti-atherogenic cytokines such as L-5, IL-10 and IL-13. IL-4, which is a Th2 cytokine, has however pro-atherogenic properties in initial stages of atherosclerosis.[6–8] More recently, natural killer T (NKT) cells have been reported to play a role in the inflammatory process of atherosclerosis. NKT cells represent a subset of T cells expressing receptors such as NK1.1 (CD161) found on NK cells. This NK1.1 is only detected on NKT cells in C57Bl/6 mice, and not in Balb/c mice. The majority of NKT cells express a semi-invariant T cell receptor (TCR) composed of a Vα14-Jα18 α-chain paired with a Vβ8 or Vβ2 β-chain. This TCR is unique because of it's specificity for (glyco)lipid antigens. (Glyco)lipid antigens are presented by the MHC class I like molecule CD1d, which is expressed on most antigen presenting cells (APCs). Upon stimulation, NKT cells are able to produce large amounts of both Th1 cytokines (IFN-γ, IL-12 and TNF-α) and Th2 cytokines (IL-4, IL-5, IL-10 and IL-13). This make NKT cells a unique T cell population with potentially both pro- and anti-inflammatory properties.[9]

NKT cells are found in atherosclerotic plaques of both humans[10,11] and atherosclerosis-prone (LDLr$^{-/-}$ and apoE$^{-/-}$) mice.[12–15] In human lesions, NKT cells colocalize with CD1d-expressing dendritic cells (DCs) in the shoulder regions and this contributes to plaque destabilization. The NKT cells represent 2% of the total lymphocyte population within the lesion.[10,11] Recent studies show that depletion of NKT cells by crossing apoE$^{-/-}$ and LDLr$^{-/-}$ mice with CD1d$^{-/-}$ mice results in an attenuation of atherosclerosis.[12–14,16] More recently an adoptive transfer of NKT cells into RAG1$^{-/-}$LDLr$^{-/-}$ mice accelerated atherosclerosis.[17] These studies indicate that a deficiency in CD1d-dependent NKT cells accelerates atherosclerosis, and this may suggest that endogenous activation of NKT cells is a pro-atherogenic process. However, many studies on NKT cells use the synthetic glycolipid α-galactosylceramide (α-GalCer) as a ligand. Studies on a number of Th1-mediated autoimmune diseases showed that activation of NKT cells via repeated injections with α-GalCer can polarize the adaptive immune response towards a Th2 like response[18,19] Treatment with α-GalCer resulted in protection from autoimmune diabetes,[20] experimental autoimmune encephalomyelitis[21] and colitis[22] in mice. Studies on Th1-mediated atherosclerosis showed however that α-GalCer treatment, significantly increases the disease in apoE$^{-/-}$ mice.[12–14] The administration of α-GalCer (single and multiple i.p. and i.v. injections) to apoE$^{-/-}$ mice caused an increase in pro-atherogenic (IFN-γ, IL-4) cytokines within the lesion.[13,14] However, in one study, in addition to the pro-atherogenic cytokines, an increase in anti-atherogenic IL-10 was observed within the lesion[14]. In serum of apoE$^{-/-}$ mice, injected once with α-GalCer, an increase in IFN-γ, TNFα, IL-2, IL-4 and IL-5 was observed, whereas no cytokines were detected after multiple injections.[13] A recent publication shows that apoE is an important

mediator of lipid antigen presentation on CD1 molecules, which may complicate conclusions on the effect of α-GalCer activation of NKT cells on atherosclerosis in apoE$^{-/-}$ mice[23]. Therefore it would be of major interest to determine what the effect of α-GalCer activation of NKT cells is in atherosclerosis induced in LDLr$^{-/-}$ mice in combination with endogenous activation.
In this study, we show that multiple injections with α-GalCer reduced atherosclerosis in LDLr$^{-/-}$ mice but was not protective in apoE$^{-/-}$ mice. ApoE$^{-/-}$ splenocytes showed a lower proliferative response towards α-GalCer and showed a dampened cytokine production, both *in vitro* and *in vivo*. Both effects may be caused by the lack of apoE. In addition, Western-type diet feeding of LDLr$^{-/-}$ mice increased NKT cell numbers in liver and spleen. Our findings suggest that in combination with endogenous activation of NKT cells, treatment with α-GalCer can be protective against atherosclerosis.

Methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr$^{-/-}$ and apoE$^{-/-}$ mice were obtained from the Jacksons Laboratory as mating pairs and bred at the Gorlaeus Laboratories, Leiden, The Netherlands. Jα281$^{-/-}$ mice on a C57BL/6 background were obtained from Dr M. Taniguchi. All mice were kept under standard laboratory conditions and were fed a normal chow diet or a 'Western-type' diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). All mice used were 10-12 weeks of age at the start of the experiment. Diet and water were administered *ad libitum*.

Glycolipids

α-Galactosylceramide (α-GalCer; KRN7000) and the control analogue β-galactosylceramide (β-GalCer) were developed and manufactured by the Pharmaceutical Research Laboratory of Kirin Brewery Co. (Gunma, Japan). Both α-GalCer and β-GalCer were dissolved in water. For intraperitoneal and intravenous injections, both glycolipids were diluted in 0.9% NaCl.

Effect of α-GalCer on lesion formation

To determine the effect of α-GalCer on the initiation of atherosclerosis, atherosclerosis was induced in LDLr$^{-/-}$ and apoE$^{-/-}$ mice. The mice were fed a Western-type diet 2 weeks prior to surgery. After 2 weeks, atherosclerosis was induced by placement of perivascular collars prepared from elastic tubing (0.3 mm inside diameter; Dow Corning, Midland, Michigan), around both carotid arteries (method described by von der Thüsen et al.[24]). For this surgery the mice were anaesthetized by a subcutaneous injection of ketamine (60 mg/kg; Eurovet Animal Health), fentanyl citrate (1.26 mg/kg; Janssen Animal Health) and fluanisone (2 mg/kg; Janssen Animal Health). The diet response was followed

by measuring the cholesterol levels in serum of these mice. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Immediately after collar placement, the mice were injected twice a week with 2 μg of β-GalCer or α-GalCer. All injections were performed half i.v./half i.p. and continued for 6 weeks. Subsequently, the mice were anaesthetized by a s.c. injection with ketamine-hypnorm and exsanguinated by femoral artery transsection. The mice were perfused and fixated through the left cardiac ventricle with PBS for 15 min. and subsequently with FormalFixx for about 30 min. Common carotid arteries and both carotid bifurcations were removed for analysis as described by von der Thüsen et al.[24] The arteries were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands) and 5μm sections were made on a Leica CM 3050S Cryostat (Leica Instruments, UK) proximally of the place of collar occlusion. These cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and eosin (Merck Diagnostica, Germany). Plaque areas were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Spleen Cell Proliferation Assay

To test the responsiveness of splenocytes to α-GalCer, spleens from LDLr$^{-/-}$, apoE$^{-/-}$ and Jα281$^{-/-}$ mice were dissected and single cell suspensions were obtained by squeezing the spleen through a 70 μm cell strainer (Falcon, The Netherlands). The erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently, the splenocytes were cultured in triplicate at $2{\cdot}10^5$ cells per well of a 96-wells round-bottom plate in the presence or absence of different concentrations of α-GalCer. RPMI 1640 (with L-Glutamine) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from BioWhittaker Europe) was used as culture medium. The splenocytes were incubated for 48 hours in a humidified atmosphere (37°C; 5% CO_2). Cultures were pulsed for the final 16 hours with [6-^{3}H]-thymidine (1 μCi/well, sp. act. 24 Ci/mmol; Amersham Biosciences, The Netherlands). The amount of [6-^{3}H]-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The magnitude of the proliferative response is expressed as stimulation index (SI) defined as the ratio of the mean counts per minute of triplicate cultures with α-GalCer to the mean counts per minute in culture medium without α-GalCer.

Cytokine assays

To determine the cytokine production by splenocytes upon stimulation with α-GalCer, splenocytes were isolated from LDLr$^{-/-}$ and apoE$^{-/-}$ mice and incubated with 100 ng/ml α-GalCer in RPMI-1640 medium (supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol). After 0, 24 and 48 hours culture supernatants were collected for ELISA assays to measure the IFN-γ, IL-10 and IL-4 production conform the manufacturers protocols (eBioscience, Belgium). To determine the *in vivo*

response to α-GalCer, LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a Western-type diet and α-GalCer was injected i.p. and i.v. (50%/50%) twice a week during 7 weeks. 72 hours after the last injection the mice were sacrificed and the spleen and the mediastinal lymph nodes were dissected. Mononuclear cells were isolated using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. The cells were stimulated o/n with anti-CD3 and anti-CD28, coated onto a 96-wells plate. Cells were subsequently stained with APC-conjugated anti-CD4 or anti-CD3 (0.125 μg/sample) for 30 min and subsequently fixated and permeabilized for 20 min. Then, the cells were stained with PE-conjugated anti-IL-4 mAb, PE-conjugated anti-IL-10 mAb and PE-conjugated anti-IFN-γ mAb (eBioscience, Belgium) for 30 min. Cells were washed two times and analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Effect of Western-type diet on NKT cells

To investigate the effect of Western-type diet on the amount of NKT cells, LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a Western-type diet for 0, 1.5 and 4.5 weeks. After diet feeding, the mice were sacrificed and the liver and spleen were dissected. Mononuclear cells were isolated from these organs using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. Subsequently, the cells were stained with PerCP-conjugated CD3 and FITC-conjugated NK1.1 antibodies (eBioscience, Belgium) for 30 min. Cells were washed two times and analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Statistical analysis

All data are expressed as mean$\pm$SEM. The two-tailed student's t test was used to compare all data. P-values less than 0.05 are considered to be statistically significant.

Results

α-GalCer protects against atherosclerosis in LDLr$^{-/-}$ mice, but not in apoE$^{-/-}$ mice

Two mouse models for atherosclerosis were used to investigate the effect of multiple injection of α-GalCer to activate NKT cells on plaque formation. Male LDLr$^{-/-}$ and apoE$^{-/-}$ mice, fed a Western-type diet for two weeks, were equipped with perivascular collars around both carotid arteries to induce atherosclerosis. After surgery, diet was continued and the mice were treated by combined i.p. and i.v. injections (50%/50%) of 2 μg α-GalCer or β-GalCer per treatment. Both ligands were administered twice a week for 7 weeks. Representative examples of plaques of β-GalCer and α-GalCer treated LDLr$^{-/-}$ mice, stained with hematoxylin and eosin, are shown in figure 5.1A and 5.1B, respectively. Treatment with α-GalCer led to an 84.1% reduction in plaque size in LDLr$^{-/-}$ mice, when compared with β-GalCer treated LDLr$^{-/-}$ mice (Figure 5.1C; 16488$\pm$6286

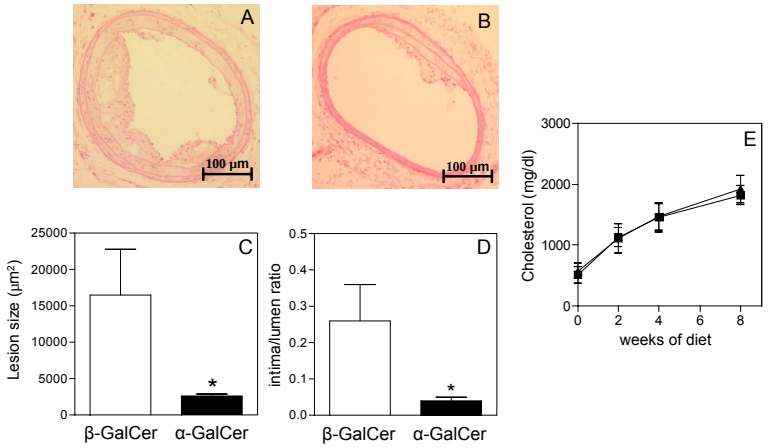

Figure 5.1: Multiple injections with α-GalCer reduces plaque formation in LDLr$^{-/-}$ mice. LDLr$^{-/-}$ mice were treated twice a week with 2 μg of α-GalCer or β-GalCer after collars were placed around the carotid arteries. Representative cryosections of plaques from β-GalCer (A) and α-GalCer (B) treated mice were stained with hematoxylin and eosin. Using computer assisted morphometric analysis plaque size (C) and intima/lumen ratio (D) were determined. During the experiment total serum cholesterol levels were monitored (E). $^*P<0.05$.

μm^2 versus 2621$\pm$263 μm^2, respectively; $P<0.05$). α-GalCer treatment also resulted in an 85.7% reduction in intima/lumen ratio (Figure 5.1D; 0.26$\pm$0.10 versus 0.04$\pm$0.01 ; $P<0.05$) and a 77.3% reduction in intima/media ratio (data not shown; 0.63$\pm$0.23 versus 0.14$\pm$0.02; $P<0.05$). Figure 5.2A and 5.2B represent examples of plaques of β-GalCer and α-GalCer treated apoE$^{-/-}$ mice, respectively. In apoE$^{-/-}$ mice a non-significant reduction on plaque size was observed (Figure 5.2C; 21700$\pm$2644 μm^2 versus 14922$\pm$3060 μm^2; P=0.30). In addition, the intima/lumen ratio (Figure 5.2D; 0.37$\pm$0.08 versus 0.25$\pm$0.04; P=0.18), and intima/media ratio (data not shown; 0.83$\pm$0.14 versus 0.64$\pm$0.07; P=0.23) were not significantly affected in apoE$^{-/-}$ mice. In both studies no effect of α-GalCer on total plasma cholesterol levels (Figure 5.1E and 5.2E) and body weight (data not shown) was observed.

In vitro effects of α-GalCer

To investigate the effect of α-GalCer on proliferation of spleen cells, splenocytes were isolated from LDLr$^{-/-}$, apoE$^{-/-}$ and Jα281$^{-/-}$ mice. Splenocytes from LDLr$^{-/-}$ mice respond to α-GalCer with a significant proliferative response. Incubation with 100 and 500 ng/ml of α-GalCer induced a 15- to 22-fold increase in proliferation (Figure 5.3, left graph, $P<0.01$). On the other hand, the response of splenocytes of apoE$^{-/-}$ mice was much lower. Incubation with 100 ng/ml of α-GalCer had no significant effect, while 500 ng/ml of α-GalCer caused a 5.2-fold increase in proliferation (Figure 5.3, middle graph, $P<0.05$). As a control, splenocytes were isolated from Jα281$^{-/-}$ mice, lacking CD1d-restricted NKT cells, and incubation of these cells with α-GalCer did not induce any proliferation of splenocytes using both concentrations of α-GalCer (Figure 5.3, right graph). Additionally, the cytokine production of splenocytes from LDLr$^{-/-}$ and apoE$^{-/-}$

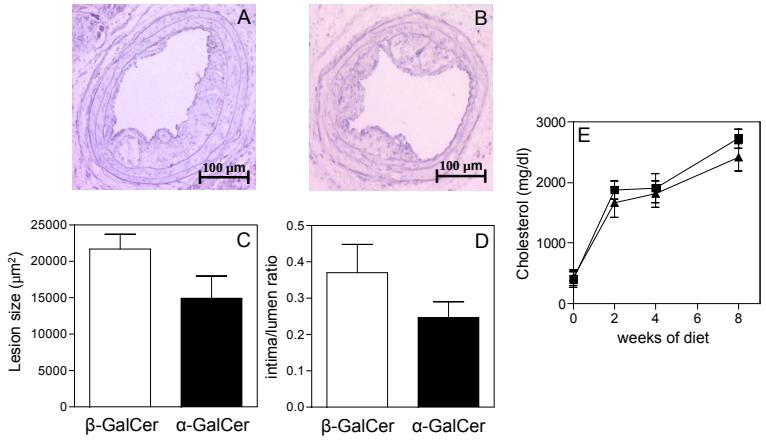

Figure 5.2: Multiple injections with α-GalCer have no significant effect on atherosclerosis in apoE$^{-/-}$ mice. ApoE$^{-/-}$ mice were treated with 2 μg of α-GalCer or β-GalCer just like the LDLr$^{-/-}$ mice in Figure 5.1. Representative cryosections of plaques from β-GalCer (A) and α-GalCer (B) treated apoE$^{-/-}$ mice were stained with hematoxylin and eosin. Plaque size (C) and intima/lumen ratio (D) were determined. During the experiment total serum cholesterol levels were monitored (E).

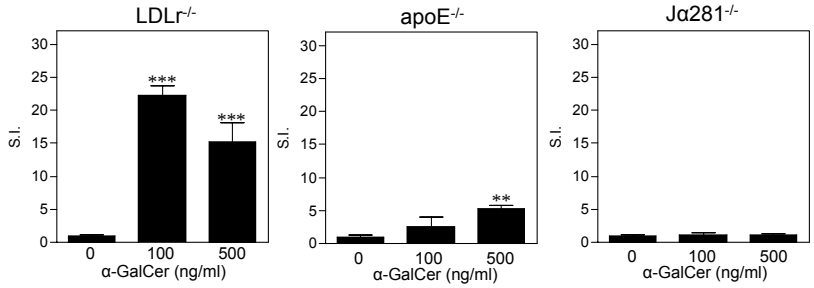

Figure 5.3: Proliferative response of splenocytes from different mouse strains on α-GalCer. Splenocytes were isolated from LDLr$^{-/-}$, apoE$^{-/-}$ and Jα281$^{-/-}$ mice and were incubated for 48 hrs with 100 or 500 ng/ml of α-GalCer. As a control, non-stimulated cells were used. Cells were pulsed with [6-^{3}H]-thymidine for the final 16 hrs and the amount of proliferation was measured. Data are shown as the stimulation index (S.I.) $\pm$ SEM. The S.I. is defined as the ratio of the mean counts per minute of triplicate cultures with α-GalCer to the mean counts per minute in culture medium without α-GalCer. $^{**}P<0.01$, $^{***}P<0.001$

mice in response to α-GalCer was determined. Stimulation of LDLr$^{-/-}$ splenocytes with 100 ng/ml of α-GalCer for 48 hours resulted in an increase in the production of IL-10 (101$\pm$13 pg/ml vs. 1559$\pm$133 pg/ml), IL-4 (36$\pm$9 pg/ml vs. 585$\pm$100 pg/ml) and IFN-γ (39$\pm$13 pg/ml vs. 381$\pm$80 pg/ml) when compared with cells cultured without α-GalCer (Figure 5.4, upper panel). Splenocytes of apoE$^{-/-}$ mice cultured with 100 ng/ml of α-GalCer for 48 hrs produced much lower amounts of IL-10 (16$\pm$1 pg/ml), IL-4 (92$\pm$22 pg/ml) and IFN-γ (9$\pm$1 pg/ml) (Figure 5.4, lower panel). When compared with splenocytes of apoE$^{-/-}$ mice cultured without α-GalCer stimulation, the relative increase in IL-4 and IFN-γ is rather high but the relative production of IL-10 is much lower when compared with the cytokine production by LDLr$^{-/-}$ splenocytes.

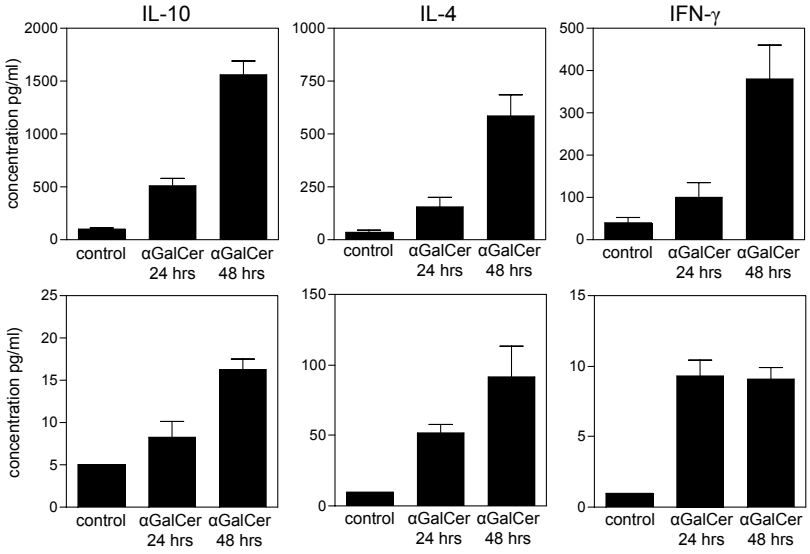

Figure 5.4: Cytokine production by splenocytes of LDLr$^{-/-}$ and apoE$^{-/-}$ mice after *in vitro* stimulation with α-GalCer. Splenocytes were isolated from LDLr$^{-/-}$ (upper panel) and apoE$^{-/-}$ (lower panel) mice and incubated with 100 ng/ml of α-GalCer or without α-GalCer (control). After 24 and 48 hrs of incubation the production of IL-10, IL-4 and IFN-γ was monitored by an ELISA on the supernatant of the cells. Values are mean cytokine concentration $\pm$ SEM.

In vivo effects of α-GalCer on cytokine production

To determine whether injections with α-GalCer affected the cytokine profile in LDLr$^{-/-}$ and apoE$^{-/-}$ mice, the cytokine production of CD3$^+$ and CD4$^+$ cells in spleen and mediastinal lymph nodes was determined after multiple injections with α-GalCer. FACS analysis showed that only a small percentage of the CD4$^+$ cells in both the spleen and the lymph nodes produce Th1 and Th2 cytokines. In β-GalCer-treated LDLr$^{-/-}$ mice, 1.22$\pm$0.22%, 0,76$\pm$0.13% and 1.20$\pm$0.13%, of the CD4$^+$ cells in the spleen produce IL-4, IL-10 and IFN-γ, respectively. There was no effect on CD4$^+$IL-4$^+$ splenocytes (1.81$\pm$0.50%; P=0.35) and CD4$^+$IFN-γ^+ splenocytes (1.66$\pm$0.28%; P=0.20) after multiple treatment of LDLr$^{-/-}$ mice with α-GalCer but a significant increase in CD4$^+$IL-10$^+$ splenocytes (2.12$\pm$0.32%; $P<$0.01) was observed (Figure 5.5A, upper panel). In the mediastinal lymph nodes, the CD4$^+$IL-4$^+$ and CD4$^+$IL-10$^+$ lymphocytes increased after multiple α-GalCer injections (0.38$\pm$0.09% vs. 1.33$\pm$0.12% and 0.46$\pm$0.12% vs. 1.42$\pm$0.11%, respectively; $P<$0.05). No effect on CD4$^+$IFN-γ^+ lymphocytes (0.52$\pm$0.06% vs. 0.56$\pm$0.12%; P=0.79) was observed (Figure 5.5A, lower panel). In the spleen of β-GalCer-treated apoE$^{-/-}$ mice, 0.84$\pm$0.21%, 1.90$\pm$0.29% and 1.31$\pm$0.16% of the lymphocytes are CD3$^+$IL-4$^+$, CD3$^+$IFN-γ^+ and CD3$^+$IL-10$^+$, respectively. There was no effect on these percentages after multiple injections with α-GalCer (1.18$\pm$0.16%, 2.50$\pm$0.18% and 1.64$\pm$0.12%, respectively) (Figure 5.5B, upper panel). In the mediastinal lymph nodes of these mice, no effect on CD3$^+$IL-4$^+$ (0.41$\pm$0.09% vs. 1.09$\pm$0.12%), CD3$^+$IL-10$^+$ (0.55$\pm$0.03% vs. 1.05$\pm$0.23%) and CD3$^+$IFN-γ^+ (2.71$\pm$0.41% to 2.28$\pm$0.29%) lymphocytes was observed after

multiple injections with α-GalCer (Figure 5.5B, lower panel).

Effect of Western-type diet feeding on NKT cell numbers

LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a Western-type diet and sacrificed at different time points. After diet feeding, the number of NKT cells in both the liver and the spleen was analyzed. After 1.5 weeks of diet feeding, the percentage of CD3^{+}NK1.1^{+} cells increased significantly from 3.24$\pm$0.14% to 6.95$\pm$0.42% ($P<0.01$) in the spleen and from 19.05$\pm$1.21% to 42.37$\pm$2.21% ($P<0.05$) in the liver of LDLr$^{-/-}$ mice, when compared with LDLr$^{-/-}$ mice sacrificed before feeding a Western-type diet. This effect was at this time point absent in the apoE$^{-/-}$ mice. In these mice there was no increase of NKT cell numbers in the spleen and a minor increase in the liver. After 4.5 weeks of diet, the percentage of NKT cells increased even more in the spleen of LDLr$^{-/-}$ mice (7.50$\pm$0.84%; $P<0.05$). In the liver of LDLr$^{-/-}$ mice, the percentage returned to control levels. In apoE$^{-/-}$ mice still no effect of Western-type diet on NKT cell numbers in the spleen was observed, whereas in the liver a 2.4-fold increase in NKT cells was observed (11.10$\pm$1.42% versus 27.18$\pm$1.50%; $P<0.05$)(Figure 5.6).

Discussion

In this study we demonstrate for the first time that α-GalCer activation of NKT cells may, depending on the experimental conditions, act in an atheroprotective manner. Activation of NKT cells via a combination of intraperitoneal and intravenous injections of α-GalCer, resulted in a 84% reduction of lesion formation in LDLr$^{-/-}$ mice in which shear-stress induced atherosclerosis was induced via collar placement around both carotid arteries and by feeding a Western-type diet. No significant effect of α-GalCer treatment was observed in the carotid arteries of apoE$^{-/-}$ mice that were also fed a Western-type diet (31% reduction, not significant). In both experiments, the β-variant of α-GalCer (β-GalCer) was used as a control ligand. This glycolipid is known to bind to CD1d but is not able to induce any proliferative response and does not affect NKT cells.[25] Several other studies showed that administration of α-GalCer to mice modulates the disease process in atherosclerosis and accelerates lesion formation. Nakai et al. showed that repeated i.p. administration of α-GalCer to apoE$^{-/-}$ mice increased atherosclerotic lesions with 67%.[12] Major et al. and Tupin et al. found a similar increased plaque size when apoE$^{-/-}$ mice, fed a normal chow diet, were treated with α-GalCer twice a week for 10 weeks.[13,14] It was hypothesized that the effect on atherosclerosis resulted from an increase in IL-4 and IFN-γ production. In a number of other Th1-mediated diseases, multiple injections of α-GalCer were shown to be protective because of the induction of Th2 cytokines IL-4, IL-5, IL-10 and IL-13 and this was beneficial in mouse models for autoimmune diseases such as diabetes[20], colitis[22] and multiple sclerosis[21], for several infectious diseases such as malaria[26] and hepatitis B[27], and for tumor treatment. Therefore it was surprising that in atherosclerosis studies, repeated injections of α-GalCer led to an increase in plaque development in atherosclerosis in apoE$^{-/-}$ mice. We observed a protective effect of α-GalCer in LDLr$^{-/-}$ mice on a Western-type

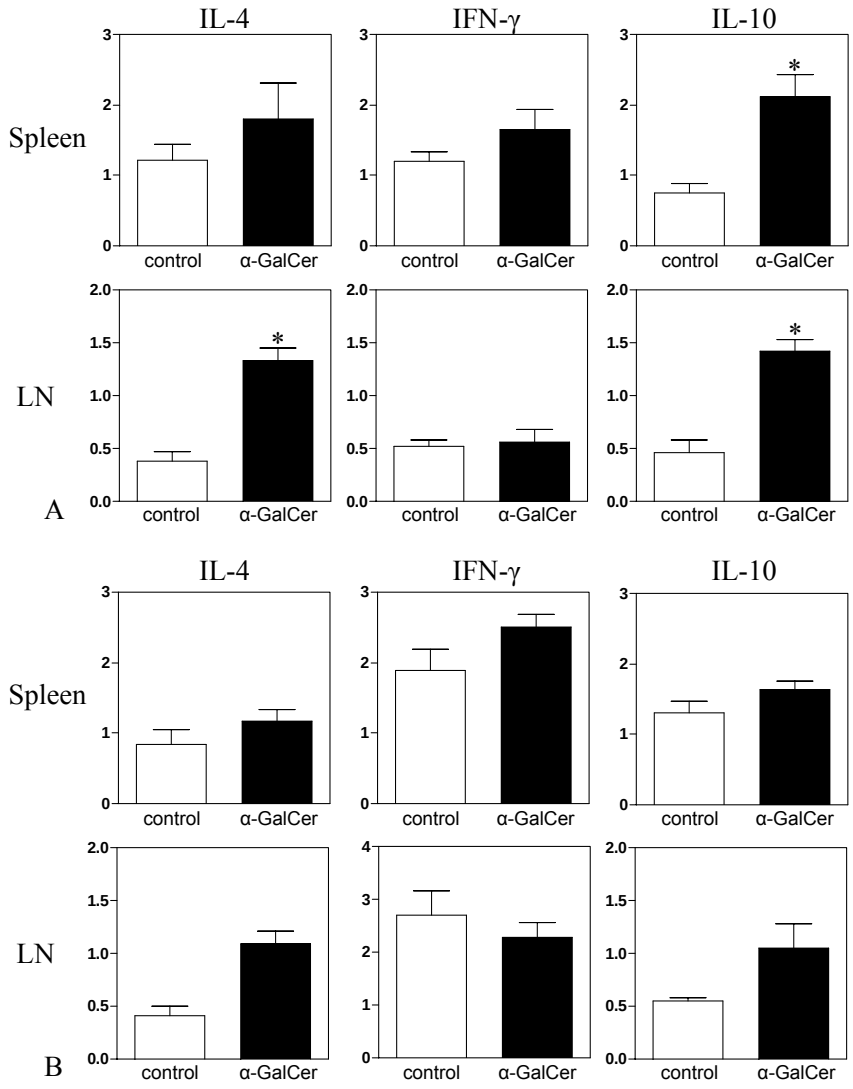

Figure 5.5: Cytokine production after *in vivo* treatment of LDLr$^{-/-}$ and apoE$^{-/-}$ mice with α-GalCer. LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a high fat diet and were treated by multiple injections of α-GalCer. After the last injection, the mice were sacrificed and the spleen and mediastinal lymph nodes were isolated. Using the FACS the percentage of CD3^{+} and CD4^{+} cells producing IL-4, IL-10 and IFN-γ within the lymphocyte population was determined. Figure A represents the LDLr$^{-/-}$ mice with on the upper side the graphs for the spleen and on the lower side for the lymph nodes. Figure B represents the apoE$^{-/-}$ mice. $^{*}P<0.05$

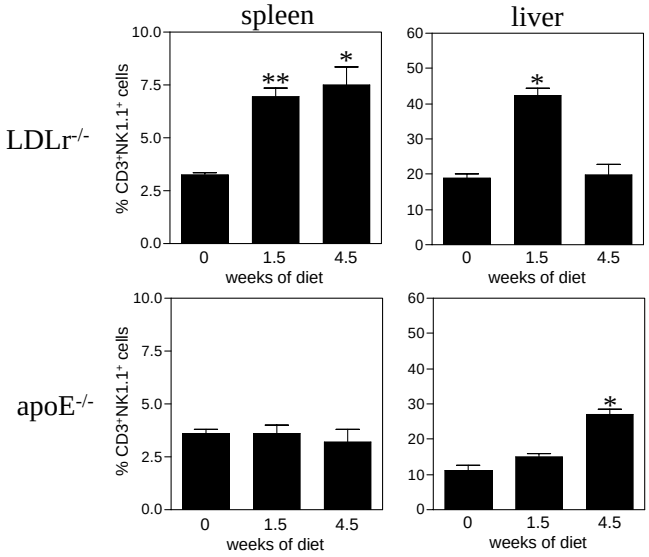

Figure 5.6: Effect of Western-type diet feeding on the number of NKT cells in LDLr$^{-/-}$ and apoE$^{-/-}$ mice. LDLr$^{-/-}$ and apoE$^{-/-}$ mice were fed a Western-type diet. After 0, 1.5 and 4.5 weeks mice were sacrificed and the number of $CD3^+NK1.1^+$ cells in the spleen and liver was measured using FACS analysis. Values are mean $\pm$ SEM. $^*P<0.05$, $^{**}P<0.01$

diet and no effect in apoE$^{-/-}$ mice on a Western-type diet, whereas previous studies showed an aggravation of atherosclerosis in apoE$^{-/-}$ mice fed a normal chow diet. A major difference between the two species is their response to α-GalCer: NKT cells from apoE$^{-/-}$ mice showed less proliferation in response to α-GalCer than those from LDLr$^{-/-}$ mice. This is explained by the study of van den Elzen et al. that shows that apoE is an important mediator in presentation of lipid antigens via CD1 molecules.[23] They proposed that apoE binds exogenous lipid antigens and efficiently targets them for receptor mediated uptake by DCs. Furthermore, intracellular apoE may be important in the delivery of the lipid to compartments containing CD1d, leading to presentation of α-GalCer on CD1d. In line with the defect in lipid presentation, apoE$^{-/-}$ mice show exacerbated experimental allergic encephalomyelitis (EAE),[28] and it may be speculated that this results from a reduced activation of sulfatide-specific CD1d-restricted NKT cells in apoE$^{-/-}$ mice, which normally inhibit EAE.[29] The lack of apoE thus explains the lower response to α-GalCer in apoE$^{-/-}$ mice compared to splenocytes from LDLr$^{-/-}$ mice which is in line with Major et al. who showed a reduced NKT cell proliferation upon α-GalCer stimulation in apoE$^{-/-}$ mice, compared with C57Bl/6 mice.[14] However, since CD1d-restricted NKT cells are still present in apoE$^{-/-}$ mice it can be concluded that apoE is not a prerequisite for NKT cell activation and other pathways can result in CD1d antigen presentation. Lipid transporters, apolipoproteins and lipoprotein receptors are also likely to participate in lipid antigen uptake and subsequent presentation via CD1 molecules.[30] This is confirmed by the low, but significant response of apoE$^{-/-}$ splenocytes to α-GalCer in our experiment and that of Major et al.[14] The lower

degree of activation of NKT cells in apoE$^{-/-}$ mice is confirmed by the fact that in our study, apoE$^{-/-}$ mice had 50% less NKT cells in the liver when compared with age-matched LDLr$^{-/-}$ mice. In addition, one publication showed that aged apoE$^{-/-}$ mice have fewer CD1d-restricted NKT cells in the spleen than young apoE$^{-/-}$ mice.[14]

In our current study we observed that splenocytes from apoE$^{-/-}$ mice produce lower amounts of cytokines in response to α-GalCer when compared with LDLr$^{-/-}$ mice. These data correlate with the lower splenocyte proliferation observed in apoE$^{-/-}$ mice and may also explain the effect of α-GalCer on atherosclerosis. Especially the relative increase in production of IL-10 after α-GalCer stimulation was much smaller in splenocytes from apoE$^{-/-}$ mice when compared with LDLr$^{-/-}$ mice, while the relative increase in production of IL-4 and IFN-γ was almost the same.

In addition, it is surprising that we did not observe an aggravating effect of α-GalCer in apoE$^{-/-}$ mice in contrast to previous publications.[12–14] This result may be caused by the fact that our mice were fed a high fat diet. As we now show in this study, Western-type diet feeding of LDLr$^{-/-}$ mice caused a rapid 2.2-fold increase in NKT cells in the liver, which returned to control levels after 4.5 weeks of diet. In spleen a 2-fold increase was observed for up to 4.5 weeks of diet which may be explained by migration of NKT cells from the liver to the spleen. After 9 weeks of diet feeding all NKT cell levels were back at basal levels (data not shown). This may be in line with the study of Aslanian et al. who showed an effect of CD1d deficiency in LDLr$^{-/-}$ mice on initial stages of atherosclerosis only. After 4 weeks of diet feeding, lesions were smaller in the CD1d$^{-/-}$LDLr$^{-/-}$ mice[16]. This is the time point at which we observed the largest increase in NKT cells. After 8 and 12 weeks of diet no effect of CD1d deficiency on atherosclerosis was observed. In apoE$^{-/-}$ mice the increase in NKT cells in the liver was delayed and in the spleen no increase was observed. This may of course result from the reduced lipid antigen presentation in apoE$^{-/-}$ mice.[30,31]

Although the natural ligand for NKT cells is still not known, our data strongly suggest that a high fat diet induces a proliferation and probably an activation of NKT cells in liver and spleen. Whether this endogenous activation of NKT cells is harmful in atherosclerosis needs further investigation, but from data on CD1d$^{-/-}$LDLr$^{-/-}$ mice we conclude that endogenous activation may accelerate atherosclerosis. In our current study the LDLr$^{-/-}$ and apoE$^{-/-}$ mice were treated with a combination of endogenous activation (diet feeding) and a synthetic ligand (α-GalCer). When α-GalCer was injected for the first time, the mice were already fed the high fat diet for two weeks. At that time point the NKT cells are triggered endogenously and present in increased levels in both liver and spleen of the LDLr$^{-/-}$ mice. We hypothesize that α-GalCer turns these "triggered" NKT cells into Th2-cytokine producing cells that ameliorate atherosclerosis. This is confirmed by our data on the in vivo cytokine profile after high fat diet feeding and multiple injections of α-GalCer. We observed a significant increase in both IL-4 and IL-10 producing T cells in the spleen and mediastinal lymph nodes of the LDLr$^{-/-}$ mice. No effect on the production of IFN-γ was observed. In apoE$^{-/-}$ mice there was however no significant effect on IL-4, IL-10 and IFN-γ production by T cells after high diet feeding and multiple injections of α-GalCer. After two weeks

of diet feeding and at the beginning of α-GalCer administration, the hepatic and splenic NKT cells of apoE$^{-/-}$ mice are not endogenously triggered by the diet yet. Administration of α-GalCer at that time point may have activated the NKT cells but was not protective. We however did not see an increased lesion size and increased levels of IL-4 and IFN-γ as was observed in former studies.[12–14] It is therefore concluded that the endogenous activation of NKT cells which is delayed in apoE$^{-/-}$ mice still affects the previously described negative effect of α-GalCer activation of NKT cells in a beneficial way, but not sufficiently enough to significantly reduce atherosclerosis.

In conclusion we describe in this study that in hyperlipidemic conditions, NKT cell activation by α-GalCer may have a protective role in atherosclerosis. However, this protective effect is only found in LDLr$^{-/-}$ mice, and not in apoE$^{-/-}$ mice since these mice have a retarded lipid antigen presentation. The proposed negative role of NKT cells in atherosclerosis may need reconsideration and further investigation into the endogenous ligands will be necessary.

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Chapter 6

Immunomodulation with OCH-pulsed dendritic cells attenuates atherosclerosis

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-Submitted for publication-

Abstract
Natural killer T (NKT) cells contribute significantly to the inflammatory response in atherosclerosis. In atheroprone apolipoprotein E (apoE) deficient mice, NKT cells have an atherogenic phenotype and their activation with the synthetic ligand α-galactosylceramide (α-GalCer) causes an increase in atherosclerotic plaque formation. Stimulation with the α-GalCer analog OCH is shown to provoke a T helper 2 (Th2) cytokine phenotype in NKT cells. In this study we observed an increased interleukin-10 (IL-10) production in $LDLr^{-/-}$ mice after intraperitoneal treatment with OCH, but no effect on atherosclerosis. Therefore we used mature dendritic cells (mDCs) to deliver the OCH to the NKT cells in the liver. Treatment with OCH-pulsed mDCs resulted in an increased number of IL-10 producing NKT cells in the liver and in a subsequent reduction in atherosclerotic plaque formation. Additionally, a reduction in cholesterol levels was observed in mice treated with OCH-pulsed DCs. Altogether, this strategy of immunomodulation with mDCs loaded with OCH may form a new therapeutical approach to prevent atherosclerosis.

Introduction

In the chronic inflammatory response that underlies atherosclerosis, both innate and adaptive mechanisms are very important.[1] Antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), are reported to initiate the autoimmune response by the uptake of autoantigens, such as oxidized low-density lipoprotein (oxLDL) and heat shock proteins. Peptides of these antigens are presented to T cells via MHC class I and II molecules.[2,3] By this, the peptides elicit the activation of T cells, especially T helper 1 (Th1) cytokine producing $CD4^+$ T cells.[4–7] T cell triggering naturally occurs within the lymphatic system, especially within the lymph nodes and spleen. After triggering, T cells migrate to the site of inflammation i.e. infiltrate the atherosclerotic plaque, where they may re-encounter their specific antigen, become activated and mediate their inflammatory damage. This is the onset of a process in which increasing numbers of immune cells are attracted to the atherosclerotic plaque and can result in occlusion of the vessels and severe cardiovascular disorders.

APCs such as macrophages and DCs are also attracted into the atherosclerotic plaque. APCs and especially the DCs express CD1 molecules, MHC class I like molecules, which specifically present lipidic antigens to T cells.[8] In mice, only CD1d, a CD1 family member, is expressed on DCs[9] and Bobryshev et al. observed that CD1d is expressed within the atherosclerotic plaque.[10] The complex of CD1d with a lipid antigen can be recognized by invariant natural killer T (NKT) cells. NKT cells are a specialized subset of T cells expressing both an invariant T cell receptor (TCR) α chain composed of Vα14-Jα18 segments and the NK cell marker NK1.1. In atherosclerosis a colocalization of NKT cells and DCs within the shoulder regions of the plaque is observed, suggesting that NKT cells are activated by antigen presentation on the DCs.[11]

NKT cells can be activated using synthetic ligands such as α-galactosylceramide[9] (α-GalCer) and the α-GalCer analog ((2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-(N-tetracosanoylamino)-1,3,4-nonanetriol) (OCH).[12,13] Activation by α-GalCer induces a rapid aspecific mixed Th1/Th2 response, in which the NKT cells produce large amounts of IL-4, IL-10, IL-12, IL-13 and IFN-γ. Recent studies showed that in contrast with other Th1-mediated autoimmune-like diseases such as autoimmune diabetes,[14–16] experimental autoimmune encephalomyelitis[17,18] and colitis,[19,20] treatment with α-GalCer accelerated the disease process in atherosclerosis-prone apoE deficient mice.[21–23] OCH, which has a truncated sphingosine chain and therefore a lower affinity for CD1d, induces a more Th2-like cytokine profile. OCH-activated NKT cells produce predominantly IL-4, IL-10 and IL-13 and low levels of IFN-γ.[12,13] This feature makes OCH an interesting glycolipid, which abrogates Th1-mediated immune responses.[12,19,24,25]

Because of their capacity to stimulate T and NKT cells, DCs are widely used in vaccination therapies. DCs may be pulsed with an antigen *ex vivo* and subsequently these ”pulsed” DCs are returned into the bloodstream. This approach is successfully used in cancer[26] and in several autoimmune diseases. DCs pulsed with bovine collagen type II protect mice from collagen-induced arthritis[27] and immature DCs (imDCs) pulsed with a peptide of glutamic acid decarboxylase protect nonobese diabetic (NOD) mice against type I diabetes.[28]

In studies on cancer it was observed that DCs pulsed with α-GalCer induced a prolonged IFN-γ-producing NKT cell response.[29,30]
In this study we observed that intraperitoneal injections of OCH had no effect on atherosclerosis despite an increased IL-10 production by splenic $CD4^+$ lymphocytes. In contrast, injection of OCH-pulsed mDCs in low density lipoprotein receptor (LDLr) deficient mice reduced atherosclerotic plaque formation which may be explained by a significant increase in IL-10^+ producing NKT cells in the liver. Additionally a significant reduction in serum cholesterol levels was observed after treatment with OCH-pulsed mDCs.

Methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male $LDLr^{-/-}$ and GFP^+ mice were obtained from Jackson's Laboratory and male C57BL/6j mice from Charles River Laboratories (Maastricht). All mice were kept under standard laboratory conditions and bred in-house. The mice were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Water and food were administered *ad libitum*.

Media and reagents

R1 cells, producing Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), and dendritic cells were cultured in IMDM (Cambrex, Belgium) supplemented with 8% FBS, 100 U/ml penicillin/streptomycin (both from PAA, Germany), 2 mM glutamax (Invitrogen, The Netherlands) and 20 μm β-mercaptoethanol (Sigma Aldrich,The Netherlands). β-GalCer was obtained from the Kirin Brewery Co Ltd, (Gunma, Japan). OCH ((2S,3S,4R)-1-O-(α-D-Galactopyranosyl)-2-(N-tetracosanoylamino)-1,3,4-nonanetriol) was synthesized as previously described by Fan et al. and dissolved in dimethyl sulphoxide (DMSO).[31] The synthesis of OCH was analyzed via nuclear magnetic resonance (NMR).

Intraperitoneal treatment with OCH

To test the direct effect of OCH on atherosclerosis, $LDLr^{-/-}$ mice were injected intraperitoneally with either 100 μg/kg β-GalCer (n=11) or OCH (n=13), twice a week for 7 weeks. Both β-GalCer and OCH were dissolved in PBS with 1% DMSO. After 3 weeks of Western-type diet feeding and prior to the treatment with β-GalCer and OCH, atherosclerosis was induced in both carotid arteries by bilateral perivascular collar placement as described previously[32]. The mice were fed a Western-type diet during the whole experiment (10 weeks). At the end of the experiment, mice were sacrificed and tissues were harvested after *in situ* perfusion with PBS and FormalFixx. Fixated tissues were embedded in OCT

compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use.

Treatment with OCH-pulsed DCs

To test the effect of OCH-pulsed DCs, bone marrow cells were harvested from the femur and tibia of C57BL/6j mice and were cultured for 10 days in complete IMDM supplemented with GM-CSF. Maturation of the DCs was accomplished via the addition of 1 μg/ml of LPS (from Salmonella Typhosa, Sigma Aldrich, The Netherlands) to the medium for 24 hours. Together with LPS, 100 ng/ml of OCH was added to a fraction of the DCs. Control DCs were incubated with LPS only. After 24 hours, the DCs were harvested and diluted in PBS. Subsequently, $LDLr^{-/-}$ mice were injected intravenously 8, 6 and 3 days before the mice were put on a Western-type diet. The mice received PBS (n=13), $1.5 \cdot 10^6$ mature DCs (n=9) or $1.5 \cdot 10^6$ OCH-pulsed mDCS (n=11). Next, the mice were either sacrificed one day or three days after the last injection with DCs or the mice were fed a Western-type diet for 3 weeks and atherosclerosis was induced in both carotid arteries by bilateral perivascular collar placement as described previously.[32] Seven weeks after collar placement, the mice were sacrificed and tissues were harvested after *in situ* perfusion with PBS and FormalFixx. Fixated tissues were embedded in OCT compound (Sakura Finetek, The Netherlands), snap frozen in liquid nitrogen and stored at -20°C until further use.

Homing of injected DCs

To investigate the homing of injected DCs, bone marrow DCs were isolated from GFP^+ mice and were cultured for 10 days in presence of GM-CSF. Subsequently, the cells were maturated with LPS (1 μg/ml) and injected intravenously in $LDLr^{-/-}$ mice fed a normal chow diet or a Western-type diet. 48 hours after injection, the mice were sacrificed and the distribution of GFP^+-DCs was determined via FACS-analysis of several organs. These organs were isolated and mononuclear cells were isolated using Lympholyte according to the manufacturers protocol (Cedarlane, Hornby, Ontario, Canada).

Histological analysis

After sacrificing the mice, the carotid arteries were sliced (5 μm) proximal of the collar and the cryosections were stained with hematoxylin (Sigma Aldrich, The Netherlands) and eosin (Merck Diagnostica, Germany). Cryosections of the aortic root (10 μm) were stained with Oil-red-O and hematoxylin to determine plaque size. Corresponding sections of carotid arteries and aortic root were stained with a macrophage specific marker (MOMA-2, Research Diagnostic Inc., New Jersey) and a collagen specific marker (Masson's Trichrome, Sigma Aldrich, The Netherlands). All images were analyzed using the Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Cholesterol and triglyceride levels

During the experiment, plasma samples obtained by tail vein bleeding were used to determine the total plasma cholesterol and triglyceride levels. Cholesterol

levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics, Germany). Triglyceride levels were quantified by using a ready-made kit (Roche Diagnostics, Germany). Precipath standardized serum (Boehringer, Germany) was used as an internal standard. Via SMART-analysis (3.2 x 30 mm, Smart System, Pharmacia) using the Superose 6 column the cholesterol distribution over different lipoproteins was analyzed.

Flow Cytometry

To check the maturation status of the DCs, cells were stained with the antibodies CD80-PE, CD86-FITC, CD40-PE, MHC II-PE, CD1d-FITC and CD11c-FITC (eBioscience, Belgium). In order to detect effects on cytokine production, 3 days after the last injection with β-GalCer or OCH, leukocytes were isolated from spleens using Lympholyte (Cedarlane, Canada). Per well of a 96-wells plate, $5{\cdot}10^5$ of these mononuclear cells were stimulated for 24 hours with αCD3/αCD28 (5 μg/ml) and 100 ng/ml OCH. Next the splenocytes were incubated with a leukocyte activation cocktail containing Golgi-stop (Becton Dickinson, CA). After 4 hours we performed intracellular staining as suggested by the manufacturers protocol. In this experiment CD4-PerCP, IFNγ-APC, IL-10-PE and IL-4-FITC antibodies were used. To determine NKT cells in blood during DC treatment, blood was collected at several time points in EDTA-coated tubes. Red blood cells were lysed using a lysis buffer containing 0.83% NH_4Cl in 0.01 M Tris/HCL (pH 7.2). Subsequently the cells were stained with CD3-PerCP and NK1.1-FITC antibodies. To detect intracellular cytokines after the DC treatment, the mice were sacrificed three days after the last injection with DCs. Leukocytes were isolated using Lympholyte (Cedarlane, Hornby, Ontario, Canada) and CD3-PerCP, NK1.1-FITC, IL-10-APC and IFNγ-APC antibodies were used to detect intracellular cytokines. All antibodies were purchased from eBioscience (Belgium). Staining of the cells was done in PBS with 1% normal mouse serum. FACS analysis was performed on a FACSCalibur (Becton Dickinson, CA). Data were analyzed with Cell Quest software.

Antibody detection

Cu-oxLDL was synthesized as described previously.[33,34] MDA-LDL was made by addition of 0.5 M MDA to 10 mg of LDL for 3 hours at 37°C. Antibodies against MDA-LDL and oxLDL were determined according to Damoiseaux et al.[35] Briefly, maxiSorp 96 well plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μg MDA-LDL or oxLDL in 100 μl PBS at 4°C. Plates were washed 5 times with 0.01 M Tris, 0.15 M NaCl and 0.05% Tween20 (pH 8.0). Mouse serum was added in duplicate at a 1:50 dilution in incubation buffer (0.1 M Tris, 0.3 M NaCl and 0.05% Tween20 (pH 8.0) overnight at 4°C. After washing, plates were incubated with either alkaline phosphatase-labelled anti-mouse IgM or IgG (Jackson Immuno-Research, Pennsylvania) both at a 1:4000 dilution in incubation buffer for 1 hour at 37°C. After washing, substrate (1 mg/ml disodium p-nitrophenyl phosphate, Sigma, The Netherlands) was added. After 2 hours at room temperature, absorbance was read at 405 nm.

Statistical analysis

Values are expressed as mean±SEM. A two tailed Student's t-test was performed to compare data. When necessary a Mann-Whitney test was performed. Probability (P) values below 0.05 were considered significant.

Results

Effect of multiple intraperitoneal injections with OCH on atherosclerosis

To investigate the effect of OCH on atherosclerosis, LDLr$^{-/-}$ mice in which atherosclerosis was induced by Western-type diet feeding and perivascular collar placement around the carotid arteries, were used. Following collar placement, β-Galactosylceramide (β-GalCer) and OCH (100 μg/kg) were administered intraperitoneally twice a week for 7 weeks. The control, β-GalCer, has a β-linkage of galactose to the ceramide group and binds to CD1d but is not able to activate NKT cells.[9] After treatment, the mice were sacrificed and the degree of atherosclerotic plaque formation in β-GalCer-treated mice and OCH-treated mice was determined in the carotid arteries (Figure 6.1A and 6.1B, respectively) and in the aortic root (Figure 6.1D and 6.1E, respectively). Treatment with OCH had no significant effect on plaque size in both the carotid arteries (Figure 6.1C; 38758±6937 μm^2 versus 39211±9363 μm^2) or the aortic root (Figure 6.1F; 438163±41923 μm^2 versus 499456±52299 μm^2) when compared with β-GalCer treated mice. During the experiment, total plasma cholesterol levels and body weight were not significantly different between both groups of mice (data not shown).

Multiple intraperitoneal injections with OCH influence the cytokine profile.

OCH is reported to induce a Th2-like cytokine profile in NKT cells. Therefore we isolated spleens of β-GalCer-treated and OCH-treated mice 3 days after the last injection with β-GalCer and OCH and performed an intracellular FACS staining to determine the cytokine profile. Following restimulation with OCH *in vitro*, the number of IFN-γ producing cells within the CD4$^+$ population of the splenocytes, decreased significantly with 34% from 1.20±0.14% in the β-GalCer-treated mice to 0.79±0.08% in the OCH-treated mice (Figure 6.2; P<0.05). Furthermore, a 76% increase in IL-10 producing cells within the CD4$^+$ population was observed (Figure 6.2; 0.76±0.14% versus 1.34±0.15%; P<0.05), while there was no effect on the number of IL-4 producing CD4$^+$ cells.

OCH priming of DCs

In order to test the effect of OCH-pulsed DCs on atherosclerosis, DCs had to be pulsed with OCH first. Therefore, bone marrow cells were isolated from the femur and tibia of C57Bl/6 mice and cultured for 10 days in presence of GM-CSF. To maturate the DCs, LPS (1 μg/ml) was added to the culture for 24 hours. The maturation status of the DCs was checked by FACS analysis. CD11c was constitutively expressed on all DCs i.e. imDCs, mDCs and OCH-pulsed

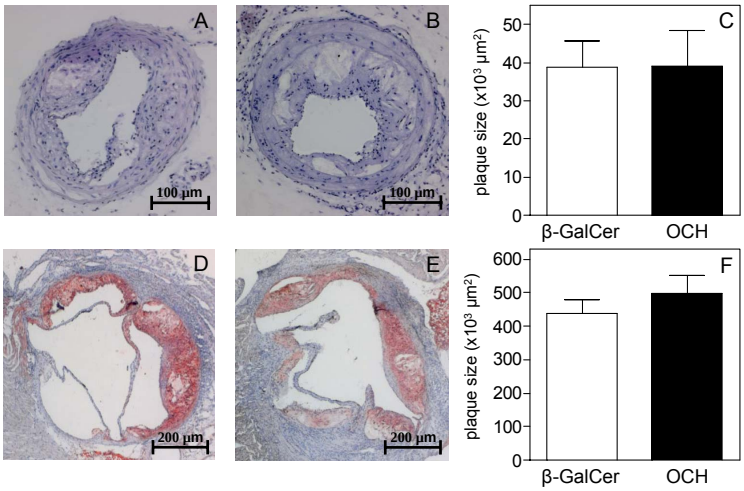

Figure 6.1: Effect of intraperitoneal injections with OCH on atherosclerotic plaque formation. Atherosclerosis was induced in LDLr$^{-/-}$ mice by Western-type diet feeding and collar placement around both carotid arteries. Immediately after collar placement, the mice were treated with β-GalCer (n=11) or OCH (n=13) twice a week. Seven weeks later, the mice were killed and the carotid arteries of β-GalCer-treated (A) and OCH-treated (B) mice were sectioned and stained with hematoxylin and eosin. The scale bar represents 100 μm. Sections of the aortic root of β-GalCer-treated (D) and OCH-treated (E) mice were stained with Oil-red-O and hematoxylin. These scale bars represent 200 μm. Lesions in the carotid arteries and aortic roots were quantified by computer assisted morphometric analysis and the plaque size was determined (C and F, respectively). Values are mean$\pm$SEM.

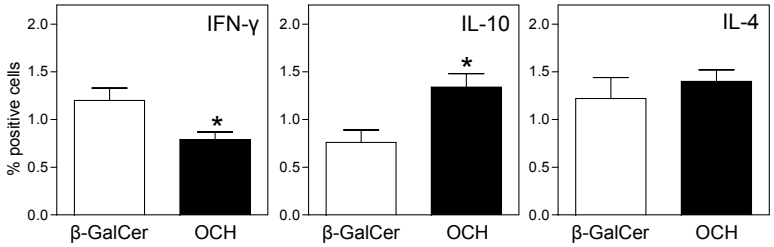

Figure 6.2: *In vivo* administration of OCH affects the cytokine profile within the spleen. After multiple treatment with OCH, spleens of LDLr$^{-/-}$ mice fed a Western-type diet and equipped with collars around both carotid arteries were dissected and splenocytes were isolated. The splenocytes of β-GalCer-treated (n=5) and OCH-treated mice (n=6) were re-stimulated with αCD3/αCD28 (5 μg/ml) and OCH (100 ng/ml) *in vitro*. After 24 hours the percentage of CD4$^+$ T cells producing IFN-γ, IL-10 and IL-4 was determined via intracellular FACS analysis after gating for CD4$^+$ lymphocytes. Values are mean$\pm$SEM. $^*P<0.05$

mDCs. CD40, CD80 and CD86, well known maturation markers for DCs are upregulated after the addition of LPS. The peptide-antigen presenting molecule MHC class II was already present on imDCs and is only slightly upregulated due to maturation, while the lipid-antigen presenting molecule CD1d is upregulated due to the addition of LPS. OCH, when added together with LPS, had no additional influence on the maturation of the DCs (Figure 6.3).

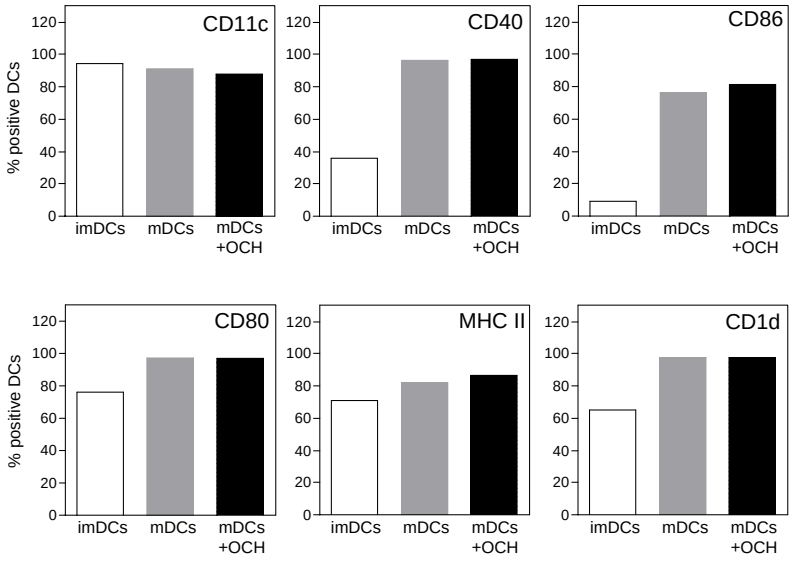

Figure 6.3: The effect of OCH on the maturation of DCs. Bone marrow derived DCs were incubated with GM-CSF for 10 days. Subsequently they were incubated with LPS (1 μg/ml) to induce maturation. Part of the DCs were simultaneously incubated with OCH to get mature OCH-pulsed DCs. After 24 hours of maturation a FACS analysis was performed and the percentage of CD11c, CD40, CD86, CD80, MHC class II, and CD1d expressing DCs was determined (white bars = imDCs, grey bars = mDCs, black bars = mDCs pulsed with OCH).

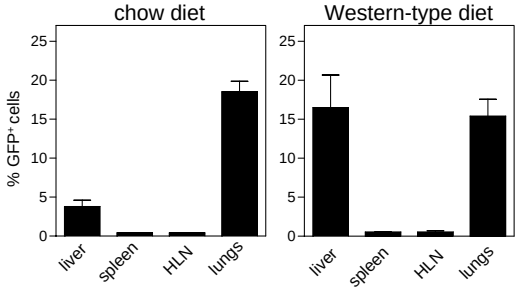

Figure 6.4: Homing of dendritic cells. DCs were isolated from bone marrow of GFP$^+$ mice and $1.5 \cdot 10^6$ of mature GFP$^+$ DCs were administered intravenously to LDLr$^{-/-}$ mice fed a chow diet (left panel, n=4) or a Western-type diet (right panel, n=4). After 48 hours the mice were sacrificed and the percentage of GFP$^+$ cells in the liver, spleen, lymph nodes around the heart (HLN) and the lungs was determined via FACS analysis. Values are mean$\pm$SEM.

Intravenously injected mDCs accumulate in several organs

To investigate the homing of intravenously injected DCs, DCs were isolated from GFP$^+$ mice and maturated by the addition of LPS. Forty-eight hours after injection of mature GFP$^+$ DCs in chow diet fed and Western-type diet fed LDLr$^{-/-}$ mice, DCs were recovered in the lung, liver, spleen and lymph nodes around the heart (HLN). When fed a chow diet 18.6% of the lymphocytes in the lung were GFP$^+$, while in the liver 3.8% was GFP$^+$. Relatively low percentages of

the injected mDCs were found in spleen and lymph nodes (Figure 4, left panel). When mice were fed a Western-type diet, 16.5% of the lymphocytes in the liver were GFP^+. The number of GFP^+ cells in other organs was not really different from that in the chow diet fed mice (Figure 6.4, right panel). Taken into account the large number of leukocytes within the liver we conclude that large numbers of the injected mDCs home to the liver.

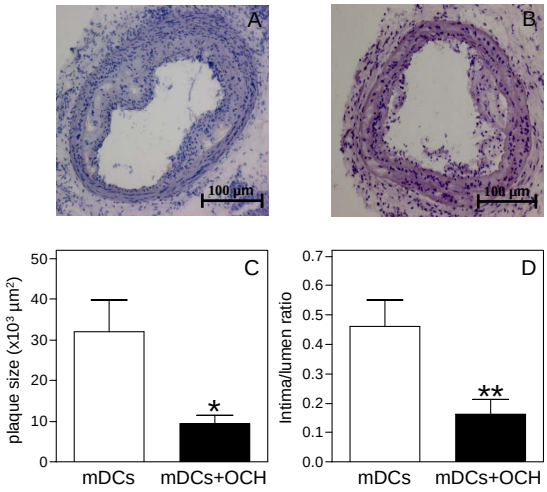

Figure 6.5: Effect of immunomodulation with OCH-pulsed mDCs on atherosclerosis in the carotid arteries. $LDLr^{-/-}$ mice were treated with mDCs or OCH-pulsed mDCs three times prior to Western-type diet feeding and collar placement around both carotid arteries. Seven weeks after collar placement, the mice were sacrificed and sections of the carotid arteries of mDC-treated mice (A, n=11) and OCH-pulsed mDC-treated mice (B, n=9) were stained with hematoxylin and eosin. Plaque size (C) and intima/lumen ratio (D) were determined by computer assisted analysis. Values are mean SEM. $^*P<0.05$ $^{**}P<0.01$

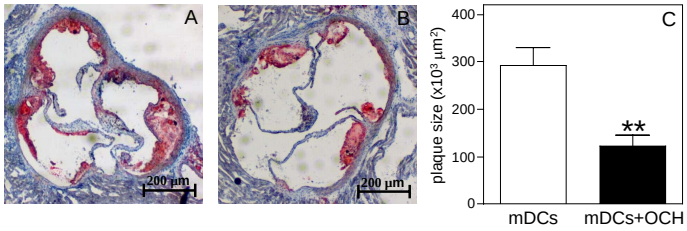

Figure 6.6: Effect of immunomodulation with OCH-pulsed mDCs on atherosclerosis at the aortic root. $LDLr^{-/-}$ mice were treated with mDCs (A, n=11) or OCH-pulsed mDCs (B, n=9) three times prior to Western-type diet feeding. After 10 weeks of Western-type diet feeding, the mice were sacrificed and sections of the aortic roots were stained with Oil-red-O and hematoxylin. The scale bars represent 200 μm. Plaque size was determined by computer assisted analysis (C). Plaque sizes are mean$\pm$SEM. $^{**}P<0.01$

Repetitive vaccination with OCH-pulsed DCs reduces atherosclerosis

To determine the effect of DCs on atherosclerosis, PBS, mDCs and OCH-pulsed mDCs were administered 3 times in 8 days intravenously to $LDLr^{-/-}$ mice. After the treatment, atherosclerosis was induced by Western-type diet feeding

and perivascular collar placement around both carotid arteries. Representative examples of hematoxylin-eosin stained atherosclerotic lesions in the carotid arteries of mDC-treated and OCH-pulsed mDC-treated mice are shown in Figure 6.5A and 6.5B, respectively. Injection of OCH-pulsed mDCs (9400±2185 μm^2) resulted in a significant 70.6% reduction in plaque size in the carotid arteries when compared to mDC-treated mice (Figure 6.5C; 31920±7914 μm^2; $P<0.05$). Injection of OCH-pulsed mDCs (0.164±0.048) also reduced the intima/lumen ratio with 64.4% (Figure 6.5D; 0.461±0.089; $P<0.01$). Additionally, the treatment with mDCs did not differ from the treatment with PBS in plaque size (48578±9231 μm2; $P=0.20$) and intima/lumen ratio (0.560±0.097; $P=0.48$) (not shown). A significant 58.1% reduction in plaque formation at the aortic root was observed when comparing OCH-pulsed mDC-treated mice (122846±21470 μm^2) with mDC-treated mice (Figure 6.6C; 293181±36193 μm^2; $P<0.01$). Representative Oil-red-O and hematoxylin stained examples of plaques at the aortic root of mDC-treated mice and mice treated with OCH-pulsed mDCs are shown in Figure 6.6A and 6.6B, respectively. Plaque size at the aortic root of PBS-treated mice (228667±32747 μm^2) was again not significantly different from the plaque size in the mDC-treated mice ($P=0.22$, not shown). Both in plaques in the carotid arteries and at the aortic root no significant changes in plaque morphology were observed. Macrophages, smooth muscle cells and collagen were present to the same extent (data not shown). Because mDC-treatment and PBS-treatment gave similar results, only the mDC treatment was used as a control for further analysis.

Increase in NKT cells due to vaccination with OCH-pulsed mDCs

During the experiment blood was withdrawn to determine the effect of vaccination with DCs on the number of NKT cells in blood. One day after the third vaccination with OCH-pulsed mDCs, which is 9 days after the start of the experiment, the percentage of $CD3^+NK1.1^+$ cells in the blood of mice treated with OCH-pulsed mDCs was significantly increased when compared with blood of mDC-treated mice (Figure 6.7A; 2.09±0.42% versus 0.94±0.24%, respectively; $P<0.05$). At the end of the experiment (day 50), the percentage of NKT cells in blood of mice treated with OCH-pulsed mDCs was still increased when compared to the mice treated with mDCs, but no significant difference was observed (1.18±0.14% versus 2.29±0.74%; $P=0.20$). To detect any changes in numbers of NKT cells in other organs we performed a new experiment in which we repeated the treatment with the mDCs and OCH-pulsed mDCs as above but now sacrificed the mice three days after the last DC-injection. FACS analysis showed that treatment with OCH-pulsed mDCs significantly increases the number of NKT cells within the liver (16.98±3.08%) when compared with the mDC-treated mice (Figure 6.7B; 8.60±0.89%; $P<0.01$). No differences in NKT cell numbers were detected in other organs. The NKT cells in the liver of mice treated with OCH-pulsed mDCs also show a Th2-like cytokine profile. The percentage of IL-10 producing NKT cells increased two-fold from 0.91±0.08% in mDC-treated mice to 1.94±0.22% in mice treated with OCH-pulsed mDCs (Figure 6.7C; $P<0.01$). In addition, the percentage of IFN-γ producing NKT cells in mice treated with OCH-pulsed mDCs (0.93±0.15%) was not significantly

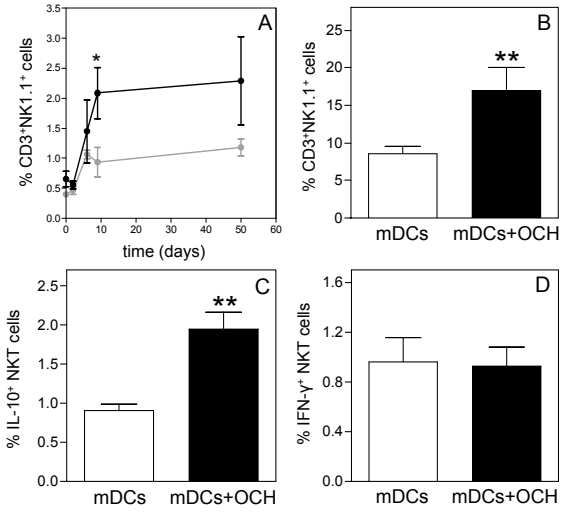

Figure 6.7: Effect of OCH-pulsed mDCs on the number and cytokine profile of NKT cells. During the experiment, blood samples were obtained via tail vein bleeding and at several critical time points the number of NKT cells was determined using FACS analysis. On the x-axis the days after the start of the experiment are depicted. The grey line represents mDC treated mice (n=3), the black one represents mice treated with OCH-pulsed mDCs (n=3) (A). In an independent experiment the mice were sacrificed three days after the last injection with DCs and the number of $CD3^{+}NK1.1^{+}$ cells in the liver of the mDC (n=5) and OCH-pulsed mDC treated mice (n=5) was determined (B). The cytokine profiles of these NKT cells were determined using an intracellular FACS protocol. The production of IL-10 (C) and IFN-γ (D) was monitored. All values are mean$\pm$SEM. $^{*}P<0.05$, $^{**}P<0.01$

different from the percentage of IFN-γ producing NKT cells in mDC treated mice (0.96$\pm$0.20%; Figure 6.7D; P=0.92).

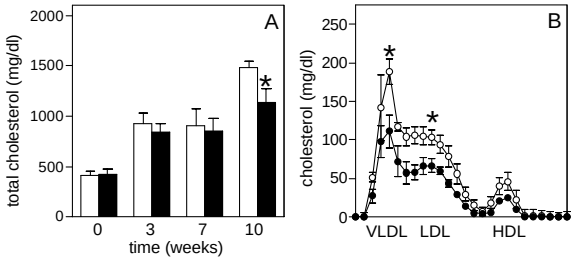

Figure 6.8: Effect of OCH-pulsed mDCs on cholesterol levels. During the experiment with immunomodulating DCs, serum samples were obtained via tail vein bleeding and the serum cholesterol levels were measured at different time points (A). 0 weeks is at the beginning of the experiment, 3 weeks is just before collar placement, 7 weeks is 4 weeks after collar placement and 10 weeks is at the end of the experiment (white bars = mDC-treated mice (n=11), black bars = mice treated with OCH-pulsed mDCs (n=9)). Serum of all mice was subsequently loaded onto a Sepharose 6 column and fractions were collected to obtain lipoprotein profiles. Fractions 3 to 7 represent VLDL, fractions 8 to 14, LDL and fractions 15 to 19, HDL (B). White dots represent the mDC-treated mice, black dots the mice treated with OCH-pulsed mDCs. Values are mean$\pm$SEM. $^{*}P<0.05$

Vaccination with OCH-pulsed mDCs reduces cholesterol levels

During the *in vivo* DC vaccination experiment, body weight, serum cholesterol and serum triglyceride levels were measured at different time points. There was no significant difference in weight due to the treatment with OCH-pulsed mDCs (data not shown). Within the first 7 weeks of the DC-vaccination experiment, also no differences in total serum cholesterol levels were observed. However, between week 7 and 10 of Western-type diet feeding a significant 23.7% lower cholesterol level was detected in mice vaccinated with OCH-pulsed mDCs (1132±136 mg/dl) when compared with mDC-treated mice (Figure 6.8A; 1483±67 mg/dl; $P<0.05$). To investigate whether this reduction in cholesterol is a reduction in cholesterol levels within a particular class of lipoproteins, a SMART analysis was performed. In serum of mice treated with OCH-pulsed mDCs, VLDL-cholesterol (111±21 mg/dl) and LDL-cholesterol (67±9 mg/dl) were significantly lower when compared with VLDL- and LDL-cholesterol levels in mDC-treated mice (Figure 6.8B; 188±17 mg/dl and 104±9 mg/dl, respectively; $P<0.05$). HDL-cholesterol was also lowered but not significantly (46±12 mg/dl versus 25±2 mg/dl; $P=0.14$). No effects were observed on triglyceride levels in serum of these mice (data not shown).

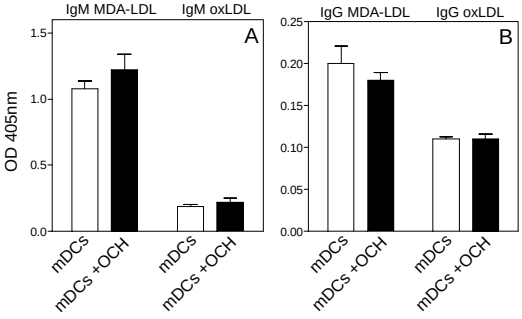

Figure 6.9: Influence of DC-treatment on MDA-LDL- and oxLDL-specific antibodies. LDLr$^{-/-}$ mice were treated intravenously with mDCs (n=11) or OCH-pulsed mDCs (n=9) and at the end of the experiment serum levels of MDA-LDL- and oxLDL-specific IgM antibodies (A) and IgG antibodies (B) were measured using a capture ELISA. Values are mean OD 405nm±SEM.

Vaccination with OCH-pulsed mDCs has no effect on antibody production

To investigate a possible role of the humoral immune response in reducing plaque formation, titers of antibodies specific for anti-modified LDL were determined. Treatment with OCH-pulsed mDCs had no influence on both malondialdehyde modified (MDA)-LDL and oxLDL-specific IgM and IgG titers in serum of the mice (Figure 6.9A and 6.9B, respectively).

Discussion

The activation of antigen specific CD4$^+$ T cells is one of the main events in the inflammatory response of atherosclerosis. Within the atherosclerotic plaque, CD4$^+$ T cells are re-activated via the recognition of specific peptide-antigens

presented by MHC class II molecules on APCs. Dendritic cells play a very important role in this process of antigen processing within the plaque. Normally, DCs are present in the intima of non-diseased arteries.[36] These DCs become activated in early stages of atherosclerosis[10,37,38] while in more advanced stages, the DCs are recruited from the adventitia[39] and blood[40] into the lesion. Within the lesions, DCs are especially located in rupture prone shoulder regions[11,39] and are found to form cell clusters with T cells. Recently, co-localization of DCs with another T cell subtype, the NKT cell, was observed within the atherosclerotic plaque.[11] The colocalization of DCs and NKT cells in the shoulder regions of the plaque,[37] and the observation of CD1d expression within the atherosclerotic plaque[10,41] suggests that the DCs present a lipidic antigen via CD1d to the NKT cells resulting in their activation. These NKT cells, present in the atherosclerotic plaque[21,22] were found to have an athero-promoting effect.[21–23]

The role of NKT cells in atherosclerosis has been studied by using the synthetic NKT cell ligand α-GalCer. Multiple intraperitoneal and/or intravenous injections of α-GalCer in atherosclerosis-prone apoE deficient mice resulted in a surprising aggravation of atherosclerosis due to the production of Th1 cytokines by NKT cells.[21–23] Especially increased levels of IFN-γ were found in these treated apoE$^{-/-}$ mice.[21,22] Studies on the effect of α-GalCer on other Th1-mediated diseases showed however that multiple injections with α-GalCer induced a more Th2-based cytokine profile of NKT cells.[14–20] Therefore it was surprising that treatment with α-GalCer resulted in an aggravation of atherosclerosis. Additionally, CD1d deficiency resulted in a reduction in atherosclerosis. LDLr$^{-/-}$ CD1d$^{-/-}$ and apoE$^{-/-}$ CD1d$^{-/-}$ mice showed a significant reduction in plaque size when compared with LDLr$^{-/-}$ mice[22,42] and apoE$^{-/-}$ mice,[21,23] respectively. From this we suggest that NKT cells in athero-prone mice are activated by certain endogenous ligands, which are still unknown and exert an athero-promoting effect. Stimulation with α-GalCer seems to worsen the situation. One explanation for this may be the increased production of IL-4, which is probably an athero-promoting Th2 cytokine.[43,44]

In this study we used OCH, another synthetic NKT cell ligand. OCH has a lower affinity for CD1d because of a shorter lipid chain and due to this lower affinity, the TCR stimulation is shorter in time than with α-GalCer. Since IFN-γ production requires a longer TCR stimulation than IL-4 and IL-10 a more pronounced Th2 cytokine profile is induced after activation with OCH. Oki et al. observed that the duration of NKT cell stimulation determines whether the NF-κB family member transcription factor c-Rel is transcribed effectively. c-Rel is identified as essential for IFN-γ production by NKT cells. They showed that c-Rel is transcribed in α-GalCer-stimulated, but not in OCH-stimulated NKT cells.[45]

In vivo administration of OCH in several mouse models is shown to abrogate Th1-mediated immune responses and to be protective against experimental autoimmune encephalomyelitis,[12] arthritis,[24] colitis[19] and diabetes.[25] In this study, OCH was administered intraperitoneally to LDLr$^{-/-}$ mice and an increased amount of IL-10 producing CD4^{+} cells was observed within the spleen. The increase in IL-10 was accompanied by a decrease in IFN-γ producing CD4^{+} cells. This cytokine profile is in line with the studies on other Th1-mediated

autoimmune disease mentioned earlier. There was however no effect on the formation of atherosclerotic lesions induced by Western-type diet feeding and collar placement around the carotid arteries of LDLr$^{-/-}$ mice. Nakai et al. showed before that injections of OCH accelerated the early phase of atherosclerosis. In that study, 8 weeks old apoE$^{-/-}$ mice, fed a regular chow diet were injected with OCH once within two weeks, three times in total. In this study they showed that the IFN-γ levels in serum of OCH-treated mice was lower than in serum of α-GalCer-treated mice while IL-4 levels were the same. This is however in contrast with other reports on OCH, because OCH is known for promoting Th2 cytokine production by NKT cells, especially when administered twice a week. Nakai et al, administered OCH once within two weeks and it is possible that this interval between two injections was too long to induce a Th2 profile. Additionally, data on IL-10 production are not presented.
An explanation for our results may be that the cytokine profile we observed was very temporal. The mice were sacrificed within 48 hours after the last injection with OCH and the induced IL-10 production together with the decreased production of IFN-γ may have been not strong enough to abrogate the Th1 response against several autoantigens in atherosclerosis. A second explanation could be an ineffective and inefficient presentation of OCH by dendritic cells to the NKT cells in the liver after intraperitoneal injections.
We therefore looked for another way to administer OCH to LDLr$^{-/-}$ mice. In several studies, the usefulness of DCs as vaccination units to impair autoimmune diseases was shown. In our previous study, mDCs pulsed with oxLDL induced a protective oxLDL specific antibody response and a reduction in atherosclerotic plaque formation in LDLr$^{-/-}$ mice (Habets et al., unpublished data). In a study by van Duivenvoorde et al. DCs were pulsed with collagen type II and this resulted in a reduction in arthritis due to a decrease in the collagen specific "Th1-associated" IgG2a response.[27] Additionally, Lo et al. showed that immature DCs pulsed with a peptide of glutamic acid decarboxylase protect NOD mice against type I diabetes.[28]
In our current study we show that mDCs injected intravenously, particularly accumulate within the liver. Dendritic cells loaded with NKT cell ligands would therefore deliver the ligand directly to the largest pool of NKT cells in the body, i.e. the liver. In the liver, the DCs can present the ligand via CD1d and induce the activation of the NKT cells. When LDLr$^{-/-}$ mice were fed a Western-type diet, even more DCs accumulated within the liver, possibly due to the elevated inflammatory status in this organ.
It was shown before that NKT cells can be expanded *in vivo* via the administration of mature dendritic cells pulsed with α-GalCer.[30] This study, performed in humans, showed a more than 100-fold increase in circulating NKT cells in all patients treated with the pulsed DCs. In two other studies a prolonged IFN-γ producing NKT cell response is induced after treatment with α-GalCer loaded mDCs.[29,46]
Because OCH induces a Th2-cytokine production by NKT cells and DCs loaded with NKT cell ligands can induce a prolonged cytokine production by NKT cells, we used this technique to study the effect of OCH-pulsed mDCs on atherosclerosis. The maturation of DCs with LPS was not affected by the

addition of OCH and the OCH-pulsed mDCs were injected in LDLr$^{-/-}$ mice. The treatment with OCH-pulsed mDCs resulted in a 70.6% decrease in atherosclerotic plaque size in the carotid arteries when compared with mDC-treated mice. The same extent of reduction was also observed at the aortic root of these mice (58.1% reduction). From our results we may suggest that the bad influence of NKT cells on atherosclerosis can be turned into an athero-protective effect, when the NKT cells are activated with OCH. The best way to deliver OCH is OCH loaded on DCs, because the drug itself was ineffective.

To investigate this athero-protective effect of NKT cells due to the DC treatment the effects on NKT cell numbers and cytokine profile were determined. Three days after the last injection with DCs, an increased number of NKT cells was observed in both blood and liver. In addition, more IL-10 producing NKT cells were found in the livers of mice treated with OCH-pulsed mDCs when compared with mDC-treated mice. The increased production of IL-10 and the unchanged production of IFN-γ could explain the reduced plaque size in these mice.[47] After activation within the liver, the NKT cells may possibly migrate out of the liver and become recruited to the atherosclerotic plaque. This migration to the plaque may explain the increased levels of NKT cells in blood after treatment with OCH-pulsed mDCs. Within the atherosclerotic plaque, the NKT cells may start excreting anti-atherogenic cytokines locally. These cytokines, such as IL-10, may influence the immune response in the vessel wall directly. It is shown before that IL-10 protects against atherosclerosis.[47–51] Another possibility is that the NKT cells may induce a bystander effect.[52,53] It is known that the bystander effects induced by activation of NKT cells with α-GalCer and OCH are different. Upon stimulation with α-GalCer, NKT cells affect the functions of other cells such as T cells, B cells, NK cells and DCs in a direct or indirect manner. Little is known about the effect of OCH-activated NKT cells on neighbouring cells but recently Oki et al. demonstrated that OCH induces less production of IFN-γ and IL-12 by bystander cells due to a lower expression of CD40L on NKT cells.[54] A bystander effect on neighbouring cells such as macrophages and T cells within the plaque may contribute further to the abrogation of the Th1 inflammatory response of atherosclerosis.

Another bystander effect of NKT cell activation in the liver may also explain the observed reduction in cholesterol levels. During the experiment serum cholesterol levels were evaluated at several critical time points. Although there was no effect on the initial cholesterol levels, treatment with OCH-pulsed mDCS induced a significant 24% reduction in cholesterol levels between week 7 and 10 of the experiment. The reduced cholesterol levels were due to a lowering of VLDL- and LDL-cholesterol, while HDL-cholesterol was unaffected. This reduction can be caused by an effect on the activity of parenchymal cells in the liver. Von der Thüsen et al. showed before that systemic IL-10 administration resulted in the lowering of VLDL and LDL cholesterol levels in LDLr$^{-/-}$ mice. In this study, the increased IL-10 production by the NKT cells in the liver may be responsible for the increased uptake of cholesterol from the blood and the subsequent secretion of cholesterol in the bile.[47]

To examine whether there are also some bystander effects of the activation of NKT cells via OCH-pulsed mDCs on the humoral immune response we

determined the effect on oxLDL and MDA-LDL specific antibodies. There were however no effects on both IgM and IgG antibody titers.
In conclusion, the activation of NKT cells via the administration of OCH-pulsed mDCs reduces the atherosclerotic plaque formation in LDLr$^{-/-}$ mice. The reduction in atherosclerosis can be explained by an increased number of IL-10 producing NKT cells in the liver. This IL-10 in the liver can induce a bystander effect that may be responsible for the lowering of cholesterol levels in the treated mice. Altogether, this strategy of immunomodulation with mDCs loaded with OCH could be used as a new therapeutical approach to prevent atherosclerosis.

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Chapter 7

Effect of deficiency in the natural killer T cell specific Vα14-Jα281 receptor on atherosclerosis in LDL receptor deficient mice

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Abstract
Natural killer T (NKT) cells, unique T cells with characteristics of NK cells, are activated by lipid antigens presented by CD1d. Thus far a few CD1d-binding plant- and bacteria-derived glycolipids are found to be natural ligands for NKT cells but their exact role is unclear. Since atherosclerosis is a disease in which lipids play a predominant role and since CD1d deficiency reduces atherosclerosis, one could imagine that one of those lipids might be an endogenous ligand for NKT cells. LDLr$^{-/-}$ mice were fed a Western-type diet for 0-24 weeks and during this period the frequency of NKT cells firstly increased in the liver and subsequently in spleen and lymph nodes. LDLr$^{-/-}$ mice were crossbred with Jα281$^{-/-}$ mice, deficient in NKT cells, and fed a Western-type diet for 12 weeks. Lesion size at the aortic root was unaffected in Jα281$^{-/-}$LDLr$^{-/-}$ mice when compared to LDLr$^{-/-}$ mice. Interestingly, splenocytes from Jα281$^{-/-}$ mice, which do not respond to α-GalCer stimulation, showed an impaired proliferation upon stimulation with oxLDL as compared to LDLr$^{-/-}$ mice. To conclude, NKT cells do not play a role in later stages of atherosclerosis. OxLDL, which is one of the most important (modified) lipids in atherosclerosis, or one of its components, may be an endogenous ligand for NKT cells but does not utilize the activation of NKT cells to affect advanced lesions in atherosclerosis.

Introduction

Natural killer T (NKT) cells represent a small population of T cells, which share characteristics with NK cells. A majority of the NKT cells express NK1.1 and a TCR composed of the Vα14-Jα18 α-chain paired with a Vβ8 or Vβ2 β-chain.[1,2] This TCR is different from the normal TCRs because it recognizes glycolipid antigens, which are presented to NKT cells by CD1d, an MHC class I related molecule present on most antigen presenting cells (APCs). α-Galactosylceramide (α-GalCer) was the first described glycolipid presented by CD1d to NKT cells and able to induce their proliferation.[3–8] Most studies on NKT cells use this ligand or one of its analogues to activate NKT cells. α-GalCer stimulation of NKT cells results in an enormous secretion of both Th1 and Th2 cytokines.[5–9] Repetitive administration of α-GalCer is shown to protect against several autoimmune diseases,[10–12] however several studies in apoE$^{-/-}$ mice showed that α-GalCer activated NKT cells accelerate atherosclerotic plaque formation.[13–15] Deficiency in CD1d, which leads to a lack of CD1d dependent NKT cells, also resulted in an amelioration of atherosclerosis in both apoE$^{-/-}$ and LDLr$^{-/-}$ mice.[13–16] These data suggested that NKT cells aggravate atherosclerosis and it also indicates that NKT cells are activated by endogenous ligands during the onset of atherosclerosis. In these studies, experiments were performed with CD1d$^{-/-}$ mice. These mice lack CD1d-restriced NKT cells but are also deficient in other T cells that are activated via the presentation of glycolipids or hydrophobic peptides presented by CD1d.[17–19] Another problem is that CD1d-independent NKT cells are still present in these mice. To avoid this problem, Jα281$^{-/-}$ mice are used in this study. These mice are deficient in both CD1d-restricted and CD1d-non-restricted NKT cells, while other CD1d-dependent T cells are unaffected.

X-ray data revealed that the lipid portion of ligands for NKT cells fits in a CD1d-binding groove whereas the carbohydrate part is exposed for recognition by the TCR of NKT cells.[20,21] Synthetic variants of α-GalCer showed that also sulfatides bind to CD1d and effectively stimulate NKT cells.[22] A few natural ligands for NKT cells are known nowadays. Recently some microbial ligands were found. Glycosphingolipids present in the outer membrane of *Sphingomonas* strongly activate NKT cells and NKT cells are important for the clearance of this infection.[23–25] In addition, more recently a self-ligand for NKT cells was found. The endogenous lysosomal glycosphingolipid isoglobotrihexosylceramide (iGb3) may activate mouse Vα14 and human Vα24 NKT cells and seems to be important for the intra-thymic development of NKT cells,[26,27] while a role for iGb3 in activation of peripheral NKT cells has also been described.[23] Others showed that plant-derived glycolipids may be natural CD1d-binding ligands for NKT cells.[28] The active plant- and bacteria-derived glycolipids are especially phosphatidyl choline (PC) and phosphatidyl ethanloamin (PE) or glycolipids that have lipid moieties which are almost identical to PC and PE.[28,29] Whether these ligands affect the Th1/Th2 cytokine secretion of NKT cells and whether these ligands can be used as treatment for diseases needs further investigation.[30]

Atherosclerosis is a disorder in which enhanced plasma lipid levels play a central role. Because the TCR of NKT cells recognizes lipid antigens presented on CD1d it is plausible that one of the lipids in atherosclerosis may be an endogenous

ligand for NKT cells. Among these lipids, phospholipids and triglycerides are mainly bound to proteins for transport: the lipoproteins.
In this study we observed an increased frequency of NKT cells in several organs after Western-type diet feeding of LDLr$^{-/-}$ mice. In addition we showed that oxLDL may be an endogenous ligand for NKT cells since NKT cell deficient splenocytes did not proliferate in response to oxLDL when compared to LDLr$^{-/-}$ splenocytes. Deficiency in NKT cells at advanced stages of atherosclerosis did not affect lesion size and therefore we conclude that NKT cells play a pro-atherogenic role only in initial stages of atherosclerosis.

Materials and Methods

Animals

All animal work was approved by the regulatory authority of Leiden University and carried out in compliance with the Dutch government guidelines. Male LDLr$^{-/-}$ mice on a total C57BL/6 background were obtained from Jackson Laboratory, Bar Harbor, Me. Jα281$^{-/-}$ mice on a C57BL/6 background were obtained from Dr M. Taniguchi. Double deficient LDLr$^{-/-}$Jα281$^{-/-}$ mice were generated by crossing Jα281$^{-/-}$ mice with the LDLr$^{-/-}$ mice. The offspring was intercrossed to produce mice with homozygous deletion in both LDLr and Jα281 genes (LDLr$^{-/-}$Jα281$^{-/-}$). For experiments, 10-12 week old male LDLr$^{-/-}$, Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were used. All mice were kept under standard laboratory conditions and bred in-house. The mice were fed a normal chow diet or a Western-type diet containing 0.25% cholesterol and 15% cocoa butter (Special Diet Services, Witham, Essex, UK). Water and food were administered *ad libitum*.

Media and reagents

The complete cell culture medium used for splenocytes was RPMI 1640 (with L-Glutamine) (Cambrex, Belgium) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from BioWhittaker Europe) was used as culture medium. LDL was isolated from serum of a healthy volunteer after centrifugation of the serum according to Redgrave et al.[31] The isolated LDL was dialyzed against phosphate buffered saline (PBS) with 10 μM EDTA (pH 7.4) for 24 hours at 4°C and oxidized by exposure to 10 μM $CuSO_4$ at 37°C for 20 hours as previously described.[32]

Effect of Western type diet feeding on NKT cells

To investigate the effect of Western-type diet on the number of NKT cells, LDLr$^{-/-}$ mice were fed a Western-type diet for 0, 1.5, 4.5, 9 and 24 weeks. After diet feeding, the mice were sacrificed and the liver, spleen, mediastinal lymph nodes and iliac lymph nodes were dissected. Also blood was collected from these mice. Mononuclear cells were isolated from spleen, liver and blood using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. The lymph nodes were squeezed through a 70 μm cell strainer and a single cell suspension

was used. Subsequently, the mononuclear cells were stained with PerCP-conjugated CD3 and FITC-conjugated NK1.1 antibodies (eBioscience, Belgium) for 30 min. To block a-specific binding of cytokines, samples were incubated with normal mouse serum for 30 min. before staining. After staining, the cells were washed two times and analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Splenocyte proliferation

To test the responsiveness of splenocytes to α-GalCer, spleens from LDLr$^{-/-}$, Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were dissected and single cell suspensions were obtained by squeezing the spleen through a 70μm cell strainer (Falcon, The Netherlands). The erythrocytes were eliminated by incubating the cells with erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3). Subsequently, the splenocytes were cultured in triplicate at $2{\cdot}10^5$ cells per well of a 96-wells round-bottom plate in complete medium with or without 100 ng/ml or 500 ng/ml of α-GalCer. The splenocytes were incubated for 48 hours in a humidified atmosphere (37°C; 5% CO_2). Cultures were pulsed for the final 16 hours with [6-^{3}H]-thymidine (1 μCi/well, sp. act. 24 Ci/mmol; Amersham Biosciences, The Netherlands). The amount of [6-^{3}H]-thymidine incorporation was measured using a liquid scintillation analyzer (Tri-Carb 2900R). The magnitude of the proliferative response is expressed as stimulation index (SI) defined as the ratio of the mean counts per minute of triplicate cultures with α-GalCer to the mean counts per minute in culture medium without α-GalCer. To investigate the response of different splenocytes to oxLDL, spleens were dissected from LDLr$^{-/-}$ and Jα281$^{-/-}$ mice. Splenocytes were isolated as above and cultured with 0, 0.5 or 1 μg/ml of oxLDL for 24 hours.

FACS analysis

To verify the deficiency in NKT cells in the LDLr$^{-/-}$Jα281$^{-/-}$ mice, livers were isolated from both LDLr$^{-/-}$Jα281$^{-/-}$ and LDLr$^{-/-}$ mice. Mononuclear cells were isolated from the non-parenchymal cell population in the liver using Lympholyte (Cedarlane, Ontario, Canada) conform the manufacturers protocol. Subsequently, the cells were stained with APC-conjugated CD3 and PE-conjugated α-GalCer/CD1d tetramer (NIH tetramer core facility, GA) for 30 min. Cells were washed two times and analyzed immediately by flow cytometry on a FACSCalibur. All data were analyzed with CELLQuest software (BD Biosciences, The Netherlands).

Induction of atherosclerosis

To determine the effect of a deficiency in NKT cells on atherosclerosis, atherosclerosis was induced in LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice. The mice were fed a Western-type diet for 12 weeks. The diet response is followed by measuring the cholesterol levels in serum of these mice. Total cholesterol levels were quantified spectrophotometrically using an enzymatic procedure (Roche Diagnostics,

Germany). After 12 weeks, the mice were anaesthetized by a *s.c.* injection with ketamine-hypnorm and exsanguinated by femoral artery transsection. The mice were perfused and fixated through the left cardiac ventricle with PBS for 15 min. and subsequently with FormalFixx for about 30 min. The hearts with the aortic root were removed and were embedded in OCT compound (TissueTek; Sakura Finetek, The Netherlands). 10μm cryosections of the aortic root were made on a Leica CM 3050S Cryostat (Leica Instruments, UK). These cryosections were stained with hematoxylin (Sigma Diagnostics, MO) and Oil-red O. Plaque areas were measured using a Leica DM-RE microscope and LeicaQwin software (Leica Imaging Systems, UK).

Statistical analysis

All data are expressed as mean$\pm$SEM. The two-tailed student's t-test was used to compare all data. *P*-values less than 0.05 are considered to be statistically significant.

Results

Increased NKT cell frequency after Western-type feeding

LDLr$^{-/-}$ mice were fed a Western-type diet for 0, 1.5, 4.5, 9 or 24 weeks. Subsequently, the mice were euthanized and the frequency of NKT cells was determined in several organs. After 1.5 weeks of high fat diet, an increase in CD3$^+$NK1.1$^+$ cells was observed in the liver (19.05$\pm$1.21% versus 42.37$\pm$2.21%; $P<0.05$) and the spleen (3.24$\pm$0.14% versus 6.95$\pm$0.42%; $P<0.01$), compared to mice sacrificed before high fat diet feeding was started. This increase persisted in the spleen after 4.5 weeks of diet feeding (7.50$\pm$0.84%; $P<0.05$), whereas the frequency of CD3$^+$NK1.1$^+$ cells decreased to basal level again in the liver. This remained for an additional 20 weeks of high fat diet feeding (Figure 7.1, upper part). After 9 weeks, an increase in CD3$^+$NK1.1$^+$ cells was also observed in the mediastinal lymph nodes, located near the ascending aorta and the heart (5.85$\pm$0.98% versus 8.97$\pm$0.60%; $P<0.05$). After 24 weeks of high fat diet feeding, the frequency of CD3$^+$NK1.1$^+$ cells in all organs returned to basal level like before the mice were fed a high fat diet. During these 24 weeks there was no significant effect on the frequency of CD3$^+$NK1.1$^+$ cells in blood and iliac lymph nodes (Figure 7.1, lower part).

NKT cell response to α-GalCer and oxLDL

To investigate whether oxLDL could be a ligand for NKT cells, splenocytes were isolated from LDLr$^{-/-}$, Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice. As a control, the responsiveness of splenocytes from these mice to α-GalCer was tested first. Splenocytes from LDLr$^{-/-}$ mice showed a large response to 100 and 500 ng/ml of α-GalCer; stimulation indexes of 22.2$\pm$1.4 and 15.2$\pm$2.9 ($P<0.001$), respectively. Splenocytes from the NKT cell deficient Jα281$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were inert to α-GalCer. Splenocyte populations lacking NKT cells do not show

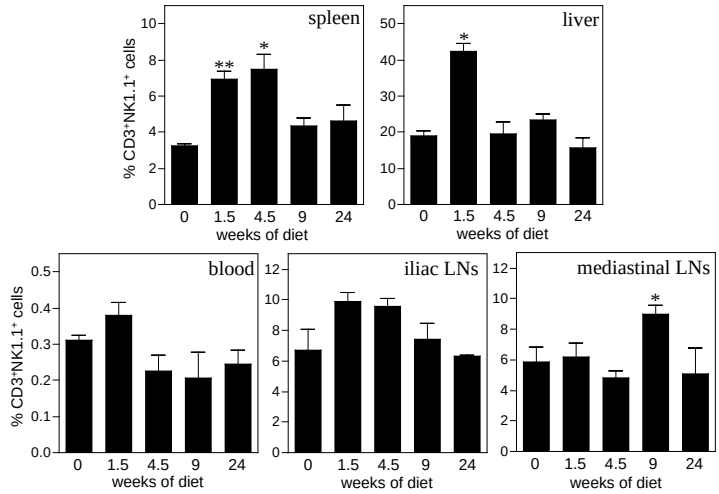

Figure 7.1: NKT cell frequency in different organs after high fat diet feeding. LDLr$^{-/-}$ mice were fed a Western-type diet for 1.5, 4.5, 9 or 24 weeks. Subsequently, the mice were sacrificed and the number of CD3^{+}NK1.1^{+} cells in the spleen, liver, blood, iliac lymph nodes and mediastinal lymph nodes was measured using FACS analysis. As a control, mice were sacrificed before high fat diet feeding was started (indicated as 0). Values are mean $\pm$ SEM. $^{*}P<0.05$, $^{**}P<0.01$

any significant proliferative response to α-GalCer (Figure 7.2). To investigate whether oxLDL could be a ligand for NKT cells, splenocytes were isolated from LDLr$^{-/-}$ mice and Jα281$^{-/-}$ mice and were incubated with different concentrations of oxLDL. Low concentrations of oxLDL induced a 9.8 to 18.7-fold increase ($P<0.01$) in proliferation of splenocytes from LDLr$^{-/-}$ mice when compared to splenocytes incubated without oxLDL. The proliferative response of splenocytes from Jα281$^{-/-}$ mice was much lower. Only a non-significant 0.8 to 2.8-fold increase was observed and a large proliferative response was only observed at higher concentrations of oxLDL (Figure 7.3).

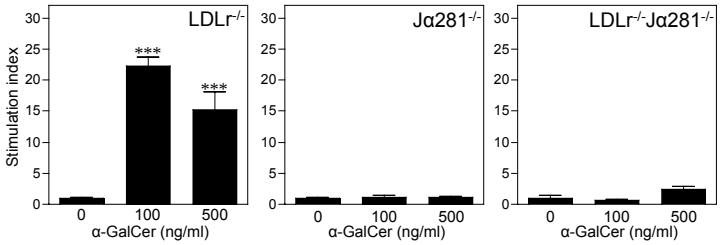

Figure 7.2: Spleen cell proliferation in response to α-GalCer. Splenocytes were isolated from LDLr$^{-/-}$, Jα281$^{-/-}$, and LDLr$^{-/-}$Jα281$^{-/-}$ mice. The splenocytes were incubated *in vitro* with or without 100 ng/ml or 500 ng/ml of α-GalCer for 48 hours. Proliferation was measured by incorporation of ^{3}H-thymidine. Data are shown as the stimulation index $\pm$ SEM. This stimulation index is the ratio of the mean cpm of cultures with α-GalCer to the mean cpm of cultures without α-GalCer. $^{***}P<0.001$.

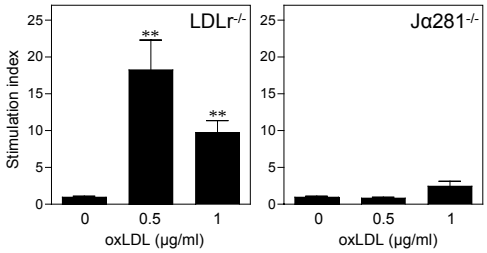

Figure 7.3: Spleen cell proliferation in response to oxLDL. Splenocytes were isolated from LDLr$^{-/-}$ and Jα281$^{-/-}$ mice. These splenocytes were incubated *in vitro* with or without 0.5 and 1 μg/ml of oxLDL for 24 hours. Proliferation was measured by incorporation of ^{3}H-thymidine. Data are shown again as the stimulation index $\pm$ SEM. The stimulation index in this experiment is the ratio of the mean cpm of cultures with oxLDL to the mean cpm of cultures without oxLDL. ** P<0.01.

Effect of NKT cell depletion on atherosclerosis

To investigate the role of NKT cells in atherosclerosis, LDLr$^{-/-}$ mice were cross-bred with Jα281$^{-/-}$ mice. These LDLr$^{-/-}$Jα281$^{-/-}$ mice were tested for the presence of NKT cells in the liver using the α-GalCer/CD1d tetramer and anti-CD3 antibodies. In the liver of LDLr$^{-/-}$ mice, 10.9$\pm$1.2% of the lymphocytes are CD3^{+}Tetramer^{+} (Figure 7.4D). These CD3^{+}Tetramer^{+} cells were absent in the LDLr$^{-/-}$Jα281$^{-/-}$ (Figure 7.4E; 1.5$\pm$0.2%). To determine whether the deficiency in NKT cells has an effect on atherosclerosis, LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice were fed a Western-type diet for 12 weeks. In figure 7.4A and 7.4B representative examples of plaques at the aortic root of LDLr$^{-/-}$ and LDLr$^{-/-}$Jα281$^{-/-}$ mice are shown, respectively. An equal level of atherosclerosis was observed at the aortic root of the LDLr$^{-/-}$ mice (623925$\pm$38750 μm^2) and LDLr$^{-/-}$Jα281$^{-/-}$ mice (Figure 7.4C; 662186 $\pm$ 49988 μm^2; P=0.58) and the lesions were rather advanced in size. In addition there was no effect on total plasma cholesterol levels and body weight during the experiment (data not shown).

Discussion

The fact that NKT cells are a unique subset of T cells recognizing lipid antigens instead of peptide antigens may provide them a unique position in atherosclerosis research, since atherosclerosis is a disease in which both natural and modified lipids play a pivotal role. Most studies on NKT cells are however performed with synthetic ligands, because only a few natural ligands are known. Recently, some bacterial, plant-derived and lysosomal glycosphingolipids which strongly activate NKT cells were found, but their role in NKT cell activation in correlation with diseases remains unclear.[23–27,30] It is of great interest to investigate the possibility whether one of the lipids important in atherosclerosis may form a natural ligand for NKT cells. In the present study we observed an increased frequency in NKT cells in the liver and spleen after 1.5 weeks of Western-type diet feeding to LDLr$^{-/-}$ mice. This increased frequency is still seen in the spleen after 4.5 weeks, whereas in the liver this frequency returned to basal level again. This can be due to a migration of NKT cells out of the liver to the spleen. There was

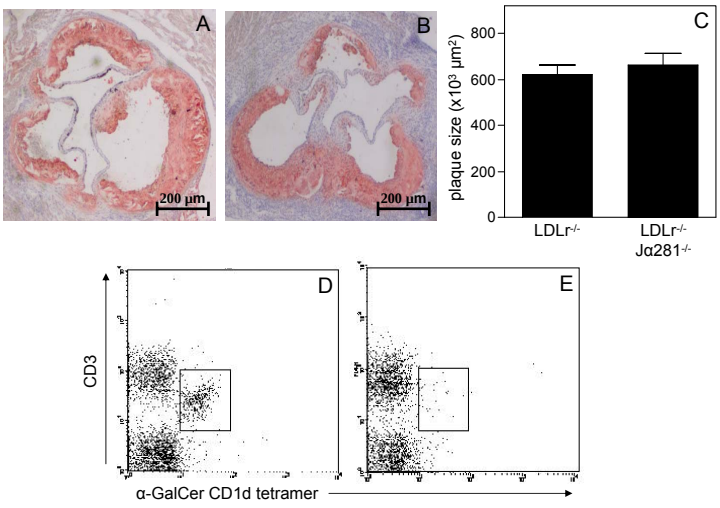

Figure 7.4: The effect of a deficiency in Jα281 on atherosclerosis. LDLr$^{-/-}$ mice were crossbred with Jα281$^{-/-}$ mice to get LDLr$^{-/-}$Jα281$^{-/-}$ mice. These LDLr$^{-/-}$Jα281$^{-/-}$ mice and LDLr$^{-/-}$ mice were fed a Western-type diet for 12 weeks. Subsequently, the mice were euthanized and sectioned aortic roots of LDLr$^{-/-}$ (A) and LDLr$^{-/-}$Jα281$^{-/-}$ (B) mice were stained with hematoxylin and Oil-red O. Lesions were quantified by computer-assisted morphometric analysis and plaque size was determined (C). To verify that the LDLr$^{-/-}$Jα281$^{-/-}$ mice lack NKT cells, a FACS analysis was performed. Lymphocytes from the liver of LDLr$^{-/-}$ (D) and LDLr$^{-/-}$Jα281$^{-/-}$ (E) mice were stained for CD3 and the α-GalCer/CD1d tetramer. Values are mean $\pm$ SEM.

however no increase in the frequency of NKT cells in blood. Whether NKT cells can migrate to the spleen via lymphatic vessels needs further investigation. The migration theory is however further supported by the observation of an increased NKT cell frequency in the mediastinal lymph nodes after 9 weeks of diet. After 24 weeks of diet the levels were the same as without diet feeding. It is however possible that the number of athero-specific NKT cells, specific for one of the lipids in the Western type diet, increased after this long period of diet feeding, but that this was not detectable because of the large decline in total number of NKT cells. The data on prolonged diet feeding (24 weeks) are in contrast with observations by Nakai et al.[15] They observed a significant decrease in both total NKT cells and α-GalCer/CD1d tetramer^{+} cells in the hepatic mononuclear cell population after 20 weeks of atherogenic diet feeding to wild type mice. They suggested that this decrease might be due to activation-induced cell death caused by for example oxLDL, due to a continuous down-modulation of NK1.1 and the TCR or due to a migration of NKT cells to other locations. Our data support this last suggestion. In addition we can confirm that NKT cells may play a role in the initiation of atherosclerosis since NKT cells seem to respond to diet feeding within the first weeks.

Since high fat diet seems to induce NKT cell proliferation and probably stimulate NKT cell migration, the next interesting question was whether oxLDL may be able to activate NKT cells. It is known that oxLDL-reactive T cells exist in the vessel wall and plaques of atherosclerosis patients.[33,34] In a previous study we observed that splenocytes from LDLr$^{-/-}$ mice respond to oxLDL in terms of an increased proliferation.[29] This indicates that a certain cell type responds to one

of the components of oxLDL. We showed that these cells were $CD3^+$ cells and not macrophages or B cells. Since oxLDL contains both proteins and lipids, epitopes of oxLDL could be both peptidic and lipidic. The macrophages and dendritic cells that ingest and process oxLDL, express different types of antigen presenting molecules such as MHC molecules and CD1d. These molecules can respectively become loaded with peptidic and lipidic antigens. It was therefore of interest to investigate if a part of the $CD3^+$ T cells proliferating in response to oxLDL could be NKT cells. Splenocytes isolated from J$\alpha281^{-/-}$ mice showed a much lower proliferative response to oxLDL when compared to splenocytes from $LDLr^{-/-}$ mice. This may indicate that NKT cells represent at least a fraction of the proliferating splenocytes when cultured with oxLDL. The observed proliferation of J$\alpha281^{-/-}$ splenocytes may be due to the proliferation of T cells responding to peptidic epitopes of oxLDL. In a recent study, VanderLaan et al. showed that LDL, isolated from the serum of $LDLr^{-/-}$ mice fed a Western-type diet, was able to induce IL-2 production by a NKT cell hybridoma when loaded on C57BL/6 DCs. This effect was absent when $CD1d^{-/-}$ DCs were used.[35] This confirms our hypothesis that NKT cells may be activated by an endogenous ligand present in the mice after Western-type diet feeding. However, they did not observe an effect of cupper-oxidized LDL on NKT cell activation. They postulated that the putative antigen in LDL was destroyed by extensive oxidation, but our results show that this may vary with isolation and the grade of oxidation of LDL. It is however clear that further investigation is needed to detect the specific structure in oxLDL that is recognized by the specific TCR on NKT cells. A few studies showed that phosphatidyl-choline (PC) may be a CD1d-binding ligand for NKT cells.[28,36] PCs are also present in oxLDL such as 1-(Palmitoyl)-2-(5-oxovaleroyl)-phosphatidyl-choline (POV-PC) and oxidized 1-palmitoyl-2-arachidonoyl-phosphatidyl-choline (oxPAPC). Both PCs trigger inflammation and promote atherosclerosis.[37–39] In future studies we will test these ligands for their possible NKT activating capacities and the effect thereof on atherosclerosis. Because NKT cells seem to play a role in atherosclerosis the effect of a deficiency in NKT cells on lesion formation was investigated. $LDLr^{-/-}$ mice were crossbred with J$\alpha281^{-/-}$ mice. These mice are deficient in both CD1d-dependent and CD1d-independent NKT cells and are different from $CD1d^{-/-}$ mice. $CD1d^{-/-}$ mice lack only CD1d-dependent NKT cells and in addition also T cells that are activated via the presentation of glycolipids or hydrophobic peptides presented by CD1d. Therefore, J$\alpha281^{-/-}$ mice are really different from $CD1d^{-/-}$ mice and J$\alpha281^{-/-}$ mice are an interesting model to investigate the exact role of NKT cells in atherosclerosis. We however observed that $LDLr^{-/-}$J$\alpha281^{-/-}$ mice fed a Western type diet for 12 weeks had similar lesion sizes as $LDLr^{-/-}$ mice. In an earlier publication,[16] it was shown that $CD1d^{-/-}LDLr^{-/-}$ mice fed an atherogenic diet for 8 and 12 weeks also have similar lesion size as $LDLr^{-/-}$ mice. In that study it was concluded that CD1d-dependent NKT cells only play a role in the initial stage of lesion development because a deficiency in CD1d reduced lesion formation in mice fed the diet for 4 weeks. In conclusion we describe in this study that the initial effect of NKT cells on atherosclerosis may be due to an activation of NKT cells by a ligand present in $LDLr^{-/-}$ mice after Western-type diet feeding. One of the candidate ligands may be oxLDL, or at least one of its components.

The exact role of activation of NKT cells by these lipids in atherosclerosis needs further investigation but we assume that these ligands activate the NKT cells which consequently accelerate atherosclerosis.

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Chapter 8

Summary and perspectives

During the last decades, more and more effort is put into the quest for a treatment of atherosclerosis, which is the major underlying pathology of cardiovascular diseases. Lipid lowering statins, anti-thrombotic drugs, and life style advice contributed to the decline in the incidence of atherosclerosis, but despite all the efforts, atherosclerosis remains the leading cause of death in the Western society. Atherosclerosis is a chronic inflammatory disease of the vasculature and is driven by a disturbed lipid metabolism and an autoimmune immune response against several autoantigens. The main aim of this thesis was to modulate the various autoimmune responses as a way to develop experimental treatments for atherosclerosis. In chapter 2 and 3, the protective role of regulatory T cells was studied. In chapter 4 and 6, dendritic cells were used in a vaccination protocol and in chapter 5, 6 and 7, the role of NKT cells in atherosclerosis was evaluated.

The most important modulators of immune responses and thus of atherosclerosis are cytokines. These cytokines can be roughly divided in two groups, the Th1 and the Th2 cytokines. The most important Th1 cytokines produced by Th1 cells are IL-12, IFN-γ, and TNF-α and these cytokines are mainly pro-atherogenic. On the other hand, most of the Th2-cytokines produced by Th2 cells (IL-5, IL10 and IL-13) are anti-atherogenic. It was long accepted that a disturbed balance between these Th1 and Th2 cells underlies the immune response in atherosclerosis. T cell responses in atherosclerosis are directed against several autoantigens. In figure 8.1 (processes I and II), the immune response to several possible antigens is shown. HSPs, oxidatively modified apoB100 and modified lipids such as oxLDL and MDA-LDL may elicit an immune response in which several cell types are important, especially the T cells. The antigens are ingested and processed by antigen presenting cells (DCs and macrophages) both within the atherosclerotic lesion and in secondary lymphoid organs such as the spleen and draining lymph nodes, which are lymph nodes near the atherosclerotic lesion (Figure 8.1, process I). The uptake of oxLDL by APCs can result in foam cell formation, both in macrophages and DCs. Normally, a DC, which has ingested an antigen maturates and migrates from the inflamed tissue towards the secondary lymphoid organs where they may induce a specific T cell or B cell response (Figure 8.1, process II). Subsequently, these activated T cells may migrate back to the inflamed tissue and to draining lymph nodes and elicit an immune response against the specific antigen, presented by the APCs in the lesion or lymph nodes (Figure 8.1, process II). In atherosclerosis, the DCs that ingest (modified) lipids and become foam cells show impaired migration to the

secondary lymph nodes.[1–3] There is however still activation of T cells specific for atherosclerotic antigens and this is due to the presence of the specific antigens through the whole body and not only within the lesion. The T cell response in atherosclerosis is mainly driven by Th1 cells, which produce IFN-γ, TNF-α and IL-12 within the lesion (Figure 8.1, process III). These cytokines are responsible for the ongoing inflammation in atherosclerosis.

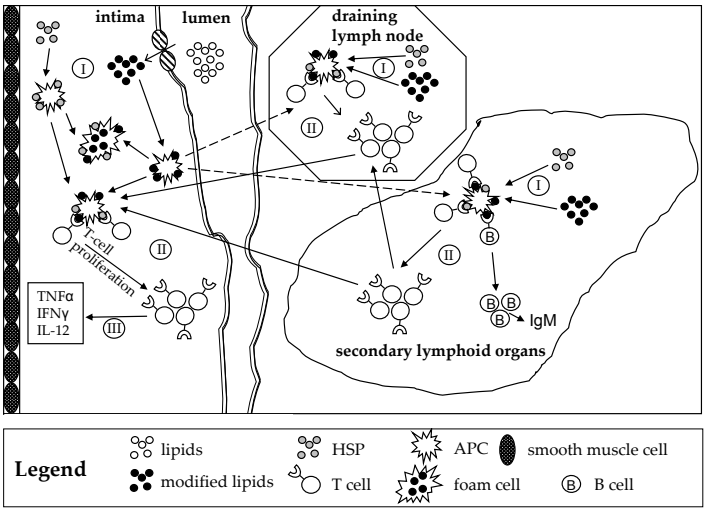

Figure 8.1: Potential sites for antigen-specific activation of T and B cells in atherosclerosis. Several critical steps are indicated by numbers: I=antigen uptake by APCs II=T and B cell activation and proliferation III=production of pro-atherogenic cytokines by Th1 cells within the atherosclerotic plaque.

Oral tolerance induction as treatment in atherosclerosis

Reducing the expression of Th1 cytokines or stimulating the production of Th2 cytokines are excessively used techniques to reduce atherosclerosis in mouse models. IL-4, considered to be a Th2 cytokine, has however pro-atherogenic properties.[4–6] Recently, the view on the disturbed balance between Th1 and Th2 cells has changed. Mallat et al. postulated that in atherosclerosis a disturbed balance exists between pathogenic Th1 and Th2 antigen-specific T cells and regulatory T cells (Tregs).[7] The Tregs mainly produce IL-10 and TGF-β, both strong anti-atherogenic cytokines. Reducing the number of Tregs accelerates atherosclerosis, whereas a stimulation and a transfer of Tregs ameliorates atherosclerosis.[7–9] Recently, Yang et al. showed that *in vitro* induced HSP60-specific Tregs prevent atherosclerotic lesion development.[10] The different types of Tregs develop in the thymus from a common ancestor. In the thymus both Foxp3$^+$ and Foxp3- Tregs T cells exist. The Foxp3$^+$ or natural Tregs migrate to the periphery and can exert their immune-suppressing properties via cell-cell contact and cytokine secretion (TGF-β and IL-10). The exact fate of natural Tregs, whether they can be antigen-specific and whether they can be activated and expand in the periphery is still not clear. In contrast with the natural Tregs, the adaptive Tregs are known for their ability to become activated in the periphery

such as in the Peyer's patches. These adaptive Tregs, which are Foxp3$^-$ when they leave the thymus, can be subdivided in Tr1 cells producing IL-10 and Th3 cells producing TGF-β. Tr1 cells do not express Foxp3, but Th3 cells may express Foxp3 upon activation[11] (Figure 8.2, process I). Adoptive Tregs may be induced via mucosal tolerance induction and may be antigen specific. In the Peyer's patches, located around the small intestines, tolerogenic DCs are present. These DCs develop especially in an environment rich in IL-10. Induction of Tregs specific for antigens in atherosclerosis was the aim of **chapters 2 and 3**. Oral tolerance is a technique that can be used to induce a Treg-response against harmful autoantigens. The fed antigens are transported into the Peyer's patches by M cells where they can be ingested by the tolerogenic DCs. This may result in the activation and proliferation of adaptive Tregs (Figure 8.2, process II). Whether natural Tregs are important in this process is not clear. In **chapter 2**, oral administration of low doses of oxLDL resulted in an increase in oxLDL-specific CD4$^+$CD25$^+$Foxp3$^+$ Tregs in both the spleen and the mesenteric lymph nodes. Tregs isolated from these lymph nodes produced more TGF-β upon *in vitro* re-stimulation with oxLDL, when compared with Tregs from control-treated mice. This indicates that the activated Tregs are most likely adaptive Th3 cells expressing Foxp3. In addition they may be natural Tregs that expand in the Peyer's patches, but this needs further investigation. The induced Tregs showed to be effective in reducing atherosclerotic plaque formation in an early and an advanced stage (Figure 8.2, processes IV and V). However, tolerance induction to MDA-LDL was not effective in inducing Tregs and consequently no effect on lesion formation was observed. The exact reason for this observation is not clear but oral tolerance induction to MDA-LDL did not induce an increase in Tregs specific for this antigen. We suggest that after uptake, MDA-LDL is not presented by the tolerogenic DCs in such a way that this leads to a proper activation of Tregs.

In the study described in **chapter 3**, HSP60 and a small peptide of HSP60 (aa 253-268) were administered orally and this resulted in an increase in CD4$^+$CD25$^+$Foxp3$^+$ Tregs in blood, Peyer's patches, mesenteric lymph nodes and spleen. The presence in blood and the decrease in Peyer's patches after two weeks indicate a migration of Tregs from the gut to the secondary lymphoid organs (Figure 8.2, process III). After re-stimulation with HSP60 *in vitro*, the splenocytes from HSP60-treated mice produced increased levels of TGF-β and IL-10 and they showed a lower HSP60-specific proliferative response when compared with splenocytes from control-treated mice. The Tregs induced after oral treatment with HSP60 ameliorated atherosclerosis in an early stage. The observation of an increased level of IL-10 upon re-stimulation may indicate that in addition to Th3 cells also Tr1 cells are induced, but these cells do not contribute to the increase in Foxp3$^+$ cells. In both studies on oral tolerance induction, an increased expression of Foxp3, CD25 and CTLA-4 in the atherosclerotic lesion indicated that the Tregs induced in the Peyer's patches may migrate to the site of inflammation i.e. the atherosclerotic lesion (Figure 8.2, process III). These data are in line with a recently published study in which oral tolerance was induced with an antigen conjugated to cholera toxin B subunit. They found several different types of Tregs that are important in oral tolerance induction i.e.

the natural Tregs but also Foxp3$^+$ and Foxp3$^-$CD25$^-$CD4$^+$ Tregs.[12] The data obtained in **chapter 2 and 3** show for the first time that the protective effect of oral tolerance induction in atherosclerosis may be caused by an activation of specific Tregs. These data support the hypothesis of Mallat et al.[7] that Tregs can counteract the pathogenic Th1 and Th2 cells specific for the same and different antigens. Within the lesion the Tregs may suppress the Th1 and Th2 response directed against the same or different antigens. This suppression may be indirect via the production of TGF-β and IL-10 (adaptive Tregs), which may result in an inhibition of the immune response against several antigens (Figure 8.2, process IV) or the suppression may be direct via cell-cell contact (natural Tregs) (Figure 8.2, process V). Normal CD4$^+$ T cells (Th1 or Th2) may upregulate the expression of the TGF-β receptor II (TβRII) after recognition of the antigen presented by APCs. Tregs specific for the same antigen also recognize the presented antigen on the APCs and start expressing surface-bound TGF-β. The subsequent interaction between TGF-β on natural Tregs and the TβRII on pathogenic T cells may result in a reduced proliferation of the pathogenic T cells (Figure 8.2, enlarged box, process V). In the future, selective elimination or inducible knockouts of Foxp3 will clarify the exact role of Tregs in atherosclerosis and tolerance induction. Furthermore, oral tolerance is a rather easy way of treatment for atherosclerosis and we will investigate whether a clinical trial is feasible.

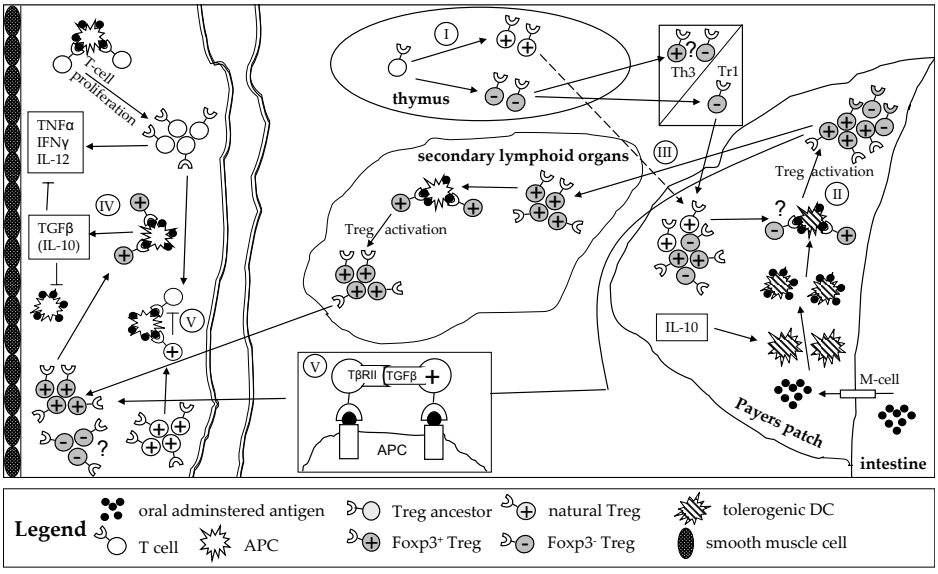

Figure 8.2: The effect of oral tolerance induction on atherosclerosis; an important role for regulatory T cells. Several critical steps are indicated by numbers: I=development of Tregs in the thymus II=activation and proliferation of adaptive Tregs III=migration of Tregs to secondary lymphoid organs and the atherosclerotic plaque IV=production of atherosclerosis-protective cytokines and the inhibition of the Th1 response within the plaque V=inhibitory effect of natural Tregs via cell-cell contact.

Dendritic cells as vaccination units in atherosclerosis

Antigen presenting cells (APCs) play a central role in immune responses and are necessary for the activation of adaptive Tregs in the gut whereas in the atherosclerotic plaque and lymphoid organs they present antigens to activate effector T cells. Dendritic cells (DCs) are the most powerful APCs. In healthy vessels only small numbers of DCs are present in the intima, immediately beneath the endothelial layer and in the adventitia.[13,14] At atherosclerosis-prone sites these DCs can start clustering in a very early stage and can elicit the immune response against autoantigens such as oxLDL and HSPs. In advanced stages, more DCs enter the lesion and clusters of DCs with T cells and NKT cells are formed in the rupture-prone shoulder regions.[1,15,16] Because of their capacity to activate T cells and NKT cells, DCs are widely used as immuno-modulating units in vaccination studies. In **chapter 4 and 6**, DCs were used to modulate two different immune responses in atherosclerosis. The DCs were isolated from the bone-marrow of C57BL/6 mice and maturated by the addition of LPS. In **chapter 4** the DCs were loaded *ex vivo* with oxLDL. This oxLDL is taken up by the DCs, shown by an Oil-red-O positive staining. OxLDL will be processed and small peptidic epitopes of oxLDL may be presented on the surface of the DCs loaded on MHC class I and II molecules. To modulate the *in vivo* immune response to oxLDL these pulsed DCs were injected in LDLr$^{-/-}$ mice (Figure 8.3, process I). After 72 hours, the injected DCs can be found especially in the lungs and the liver but also in the spleen and lymph nodes. Injections with the oxLDL-pulsed DCs resulted in an attenuation of atherosclerotic plaque formation. In addition, a decreased number of macrophages and an increase in collagen fibers was observed, contributing to a more stable lesion. OxLDL pulsed DCs also induced a significant decrease in cholesterol after 10 weeks of Western-type diet feeding. This lowering in cholesterol was accompanied by an increase in oxLDL-specific IgG antibodies which indicates that there was an activation of oxLDL specific T cells (Figure 8.3, process II). We now hypothesize that the injected DCs that homed to the spleen induced an oxLDL-specific T cell response which subsequently resulted in an activation of B cells producing oxLDL-specific IgG antibodies (Figure 8.3, process III). These antibodies may form immune-complexes with oxLDL in the circulation and this may be responsible for the reduced foam cell formation observed when macrophages were incubated with oxLDL together with serum of the DC-treated mice. A possible explanation is that the IgG antibodies form immune-complexes with oxLDL which may reduce the uptake of oxLDL via scavenger receptors on macrophages within the atherosclerotic lesion (Figure 8.3, process IV). Secondly, immune-complex formation may result in a better opsonization of oxLDL, resulting in a fast Fc-dependent removal of oxLDL from the circulation, or in neutralization of the effects of oxLDL systemically and locally which may prevent oxLDL from exerting pro-inflammatory and toxic effects in the vascular wall (Figure 8.3, process IV). The immuno-protective role of IgG antibodies was already shown in several studies in which mice were immunized with several forms of modified lipids and peptides.[17–20] The protective function is especially important because the DCs were injected before the mice were fed a Western-

type diet, and thus before oxLDL was generated. The injected oxLDL-pulsed DCs may restore the impaired migration of oxLDL-loaded DCs from the plaque to the secondary lymphoid organs, even before oxLDL was taken up by DCs within the lesion. Therefore T cells and IgG antibodies are generated that quickly respond when oxLDL is generated after high fat diet feeding. This process is like normal vaccination. Several studies already described that immunization with oxLDL, MDA-LDL or modified apoB100 peptides results in a reduction in atherosclerosis.[20–22] Major difference between this "vaccination" technique and ours is that in DC-vaccinations no adjuvant is needed. The clinical application is therefore wider than with other immunization studies. The only drawback could be that DCs from the patient itself are needed for vaccination, although these DCs can easily be generated from peripheral blood monocytes.[23]

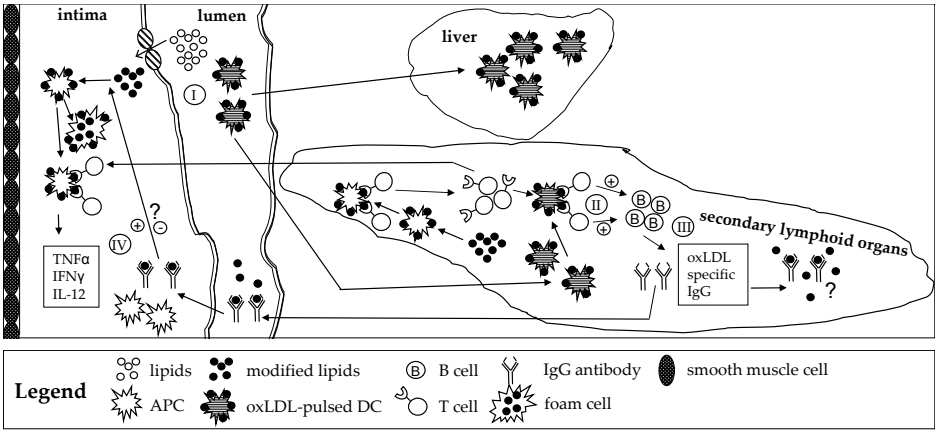

Figure 8.3: Dendritic cells pulsed with oxLDL are used to modulate the immune response in atherosclerosis. Several critical steps are indicated by numbers: I=Intravenous injection of oxLDL-pulsed DCs II=activation of oxLDL-specific T cells III=production of oxLDL-specific IgG antibodies by B cells IV=protective effect of oxLDL-specific IgG on the immune response in the atherosclerotic plaque.

NKT cell activation in atherosclerosis

In addition to MHC molecules, DCs also express CD1, another class of antigen presenting molecules. In mice only CD1d is expressed and this molecule binds glycolipid antigens instead of peptide antigens like MHC molecules do. Antigens presented by CD1d are recognized by a specialized subset of T cells, the NKT cells, which express a T cell receptor (TCR) composed of Vα14 and Jα18 (previously known as Jα281) subunits paired with a restricted set of Vβ chains. CD1d expressing DCs were found in the atherosclerotic lesions together with NKT cells.[24–27] NKT cells are known for their unique capacity to produce a diverse spectrum of cytokines, both Th1 and Th2.[28] Since many studies on other Th1 mediated diseases showed that repetitive injections with α-GalCer, which is a strong synthetic activator of NKT cells, was protective, it was expected that α-GalCer activation of NKT cells would also be protective in atherosclerosis. Accumulating data however proves that NKT cells accelerate atherosclerosis.

Depletion of CD1d by crossbreeding CD1d$^{-/-}$ mice with LDLr$^{-/-}$ and apoE$^{-/-}$ mice decreased lesion formation while activation of NKT cells by injecting α-GalCer in apoE$^{-/-}$ mice resulted in an acceleration of lesion formation.[24–26,29] NKT cells were found in atherosclerotic lesions, especially co-localized with DCs, and the general idea is that the NKT cells within the lesion are activated by an endogenous ligand. The endogenous ligand for NKT cells in atherosclerosis is however not known but since NKT cells are activated by lipidic antigens it is assumed that one of the lipids excessively present in atherosclerotic lesions and the liver may be able to activate NKT cells. It is suggested that this activation may result in Th1-biased NKT cells which may promote lesion formation (Figure 8.4, process I). In **chapters 5 and 6** we describe for the first time that NKT cells may however protect against atherosclerosis. In **chapter 5**, atherosclerosis was induced in LDLr$^{-/-}$ and apoE$^{-/-}$ mice by a combination of collar placement around the carotid arteries and feeding a Western-type diet. Subsequently, the mice were treated with α-GalCer twice a week for 7 weeks by i.v. and i.p. injections (Figure 8.4, process II). This resulted in a reduced lesion formation in LDLr$^{-/-}$ mice whereas there was no effect in apoE$^{-/-}$ mice. The difference between both mouse models is further elucidated by the observation of a lower proliferative response and a lower cytokine production in response to α-GalCer by apoE$^{-/-}$ splenocytes than LDLr$^{-/-}$ splenocytes. Additionally, the *in vivo* cytokine response in apoE$^{-/-}$ mice treated with α-GalCer was dampened when compared with LDLr$^{-/-}$ mice which showed an increased production of IL-10 and IL-4 in secondary lymphoid organs when compared with β-GalCer-treated mice. The difference in responses to α-GalCer in the two mouse models may be explained by two statements. First of all, apoE is an important mediator of lipid antigen presentation on CD1 molecules. ApoE$^{-/-}$ mice lack this important protein and consequently have an impaired ability to present lipid antigens to NKT cells (Figure 8.4, process III). This however does not explain why an increased plaque size was observed in other studies on α-GalCer treatment in apoE$^{-/-}$ mice. Another difference between our study and the other studies could however form an explanation for this. The most important difference between the studies is that our mice were fed a Western-type diet in stead of a normal chow diet. We now hypothesize that this high fat diet may influence the NKT cells before they were treated with α-GalCer. This is confirmed by the observation that a Western-type diet results in an increased NKT cell number in liver after 1.5 weeks followed by an increase in the spleen after 4.5 weeks (Figure 8.4, process IV). Due to this pre-activation of NKT cells, which may result in NKT cells that accelerate the disease (Figure 8.4, process I), α-GalCer may switch these pre-activated Th1 cytokine producing NKT cells into Th2 cytokine producing NKT cells (Figure 8.4, process V). These "double"-activated NKT cells may migrate to the lesion and secondary lymphoid organs and dampen the Th1-response via re-stimulation by endogenous ligands or α-GalCer (Figure 8.4, process VI). This may be explained by the fact that repetitive activation of NKT cells results in a more Th2-phenotype. We suggest that the repetitive injection of α-GalCer after continuous Western-type diet feeding induced a switch in cytokine-profile. In apoE$^{-/-}$ mice, a delayed response to the Western-type diet was observed. After two weeks of diet feeding and at the beginning of α-GalCer administration the hepatic and splenic NKT

cells are not endogenously triggered by the diet. NKT cells in this mouse model are activated by the injected α-GalCer but this was not protective. However, we did not observe an increase in lesion size in apoE$^{-/-}$ mice like it was observed in previous studies and therefore, it is concluded that the endogenous activation of NKT cells, which is delayed in apoE$^{-/-}$ mice, still affects the previously described negative effect of α-GalCer activation of NKT cells in a beneficial way, but not sufficiently enough to reduce atherosclerosis. From this study we can conclude that the vision on NKT cells accelerating atherosclerosis should be reconsidered. ApoE$^{-/-}$ mice are not the best model to investigate the role of NKT cells.

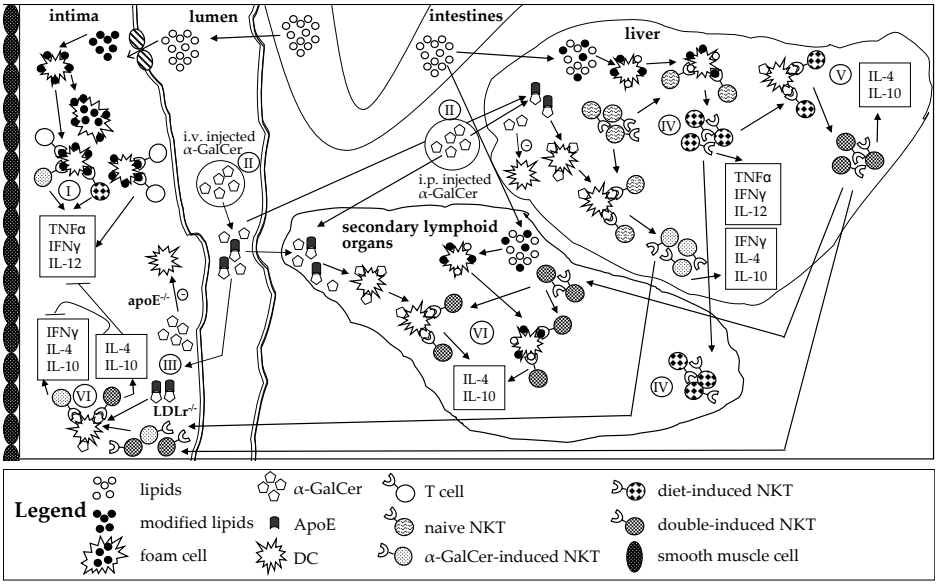

Figure 8.4: NKT cell activation via α-GalCer and its effect on atherosclerosis; an important role for high fat diet and apoE. Several critical steps are indicated by numbers: I=suggested atherosclerosis-promoting effect of NKT cell activation by lipids II=intraperitoneal and intravenous injection of α-GalCer III=lack of apoE results in an impaired ability to present lipids via CD1d to NKT cells IV=activation and proliferation of NKT cells due to a high fat diet V=proposed effect of α-GalCer; a switch from NKT cells producing Th1-cytokines to NKT cells producing Th2-cytokines VI=proposed effect of "double activated" NKT cells on the immune response in the atherosclerotic plaque.

Dendritic cells used to activate NKT cells

Ex vivo pulsed DCs can also be used as activators for NKT cells. Loading of α-GalCer on DCs and a subsequent injection of these DCs in mice and patients resulted in a prolonged activation of NKT cells producing a large amount of IFN-γ.[30,31] In **chapter 6** we used an analogue of α-GalCer, OCH, which triggers the NKT cells to a more Th2-phenotype.[32,33] OCH was administered intraperitoneally to LDLr$^{-/-}$ mice but this had no effect on atherosclerotic lesion formation. However, intravenous injection of DCs pulsed *ex vivo* with OCH (Figure 8.5, process I) reduced the lesion size dramatically. This reduction may be due to the migration of a high number of OCH-loaded DCs to the liver. There the

DCs may present OCH to the largest NKT cell pool of the body. This induced a proliferation and activation of hepatic NKT cells (Figure 8.5, process II) and these NKT cells produced more IL-10 while the same amount of IFN-γ was produced when compared with NKT cells from control-treated mice (Figure 8.5, process III). This whole process may also occur within the spleen, but this needs further investigation. After activation, the NKT cells may migrate out of the liver (and spleen) to other locations. This may explain the increased NKT cell number in the blood of mice treated with OCH-pulsed DCs. Since NKT cells are also found in atherosclerotic lesions we hypothesize that NKT cells induced in the liver migrate towards the site of inflammation. There they may be re-stimulated by OCH on injected DCs or by DCs already present in the lesion which present endogenous ligands (Figure 8.5, process IV). Upon re-stimulation the NKT cells secrete Th2 cytokines, especially IL-10, and inhibit the Th1 immune response within the lesion directly or via a bystander activation (Figure 8.4, process V). In addition, lower cholesterol levels were observed in the mice treated with OCH-pulsed DCs. This difference was only present in later stages of atherosclerosis and therefore can not explain the difference in plaque size, but it may contribute to the reduction. It is known that IL-10 influences the cholesterol levels in LDLr$^{-/-}$ mice.[34] We now suggest that the increased production of IL-10 by NKT cells in the liver stimulates the uptake of cholesterol from the blood by parenchymal cells and the subsequent secretion of cholesterol in the bile.

Endogenous ligands for NKT cells

In **chapter 5** we observed that high fat diet feeding may influence the NKT cell population in LDLr$^{-/-}$ mice. A natural endogenous ligand for NKT cells is however still not known. Recent publications suggest that some plant- and bacteria-derived glycolipids may be able to activate the NKT cells.[35,36] These glycolipids contain phosphatidyl choline (PC) and phosphatidyl ethanolamin (PE) of which the first one is also present in some lipids important in atherosclerosis; POV-PC and oxPAPC. In **chapter 7**, LDLr$^{-/-}$ mice were fed a Western-type diet and again an increase in NKT cell frequency was observed in the liver followed by the spleen and the iliac lymph nodes (Figure 8.3, process IV). These data confirm the fact that NKT cells play an important role in the initiation of atherosclerosis[29] since the increase in NKT cells is within 4.5 weeks of diet feeding. During progressive stages no differences in the number of NKT cells was observed. This is also shown by a deficiency in NKT cells, which has no effect on atherosclerosis after 12 weeks of Western-type diet feeding. OxLDL is one of the major lipids in atherosclerosis and a deficiency in NKT cells reduced the proliferative response of splenocytes to oxLDL. This indicates that oxLDL or at least one of its lipid components may be an endogenous ligand for NKT cells. We now suggest that PC units in oxLDL may be responsible for this; especially oxPAPC and POVPC are interesting candidates. These data are encouraging to continue the search for endogenous ligands in atherosclerosis. A plethora of lipids will be tested in the future which may possibly lead to a specific endogenous ligand for NKT cells. This may consequently lead to a better insight in the role of NKT cells and to new targets for therapeutic intervention in atherosclerosis. Overall, from the data in

chapters 5-7 it is suggested that NKT cells are activated by an endogenous ligand which may be one of the (PC-containing) lipids important in atherosclerosis. This activation may result in Th1-biased NKT cells accelerating atherosclerotic lesion development. The activation of the NKT cells may start in the liver, where also dendritic cells which ingest and process lipid antigens are present. The activation of NKT cells may subsequently spread through the whole body including the atherosclerotic lesion. Within the lesion they may be re-stimulated, resulting in the production of Th1 cytokines, especially IFN-γ, and this may contribute to the ongoing inflammation. However, re-stimulating the pre-activated NKT cells with α-GalCer switched the cytokine profile of NKT cells to a Th2-like profile, and they produce especially IL-10. These Th2 cytokines may dampen the Th1-mediated immune-response in atherosclerosis. This effect is only observed in LDLr$^{-/-}$ mice and is absent in apoE$^{-/-}$ mice because apoE is an important mediator of lipid antigen presentation on CD1 molecules. Activation of NKT cells by OCH, loaded on DCs, also reduced atherosclerotic lesion formation. This study was also performed in LDLr$^{-/-}$ mice that were fed a Western-type diet, further suggesting that a combination of a Western-type diet and a synthetic ligand are important to skew NKT cells to a Th2 phenotype.

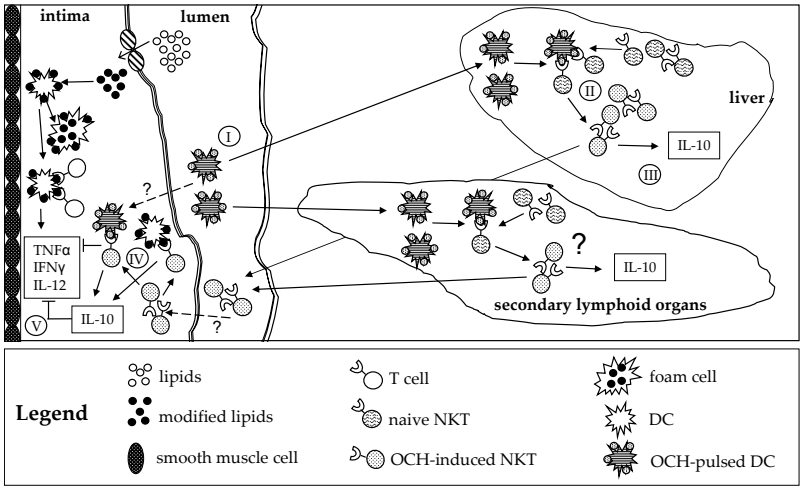

Figure 8.5: The effect of OCH-pulsed DCs on atherosclerosis; altered cytokine profile of NKT cells. Several critical steps are indicated by numbers: I=intravenous injection of OCH-pulsed DCs II=activation and proliferation of NKT cells via presentation of OCH by the injected DCs III=increased IL-10 production by NKT cells IV=restimulation of activated NKT cells within the atherosclerotic plaque by OCH or lipids presented by APCs V=proposed inhibition of the Th1-mediated immune response by IL-10 produced by the restimulated NKT cells within the atherosclerotic plaque.

Perspectives

In this thesis, several strategies to modulate the immune system during atherosclerosis have been studied in experimental therapies. These strategies provide different forms of immunotherapy and the goal of these strategies is to achieve a very efficient treatment for atherosclerosis that may be applied in

addition to the use of statins, aspirin and blood pressure lowering medication. The necessity for such as therapy is high because the current therapies in atherosclerosis are still insufficient. Statins are prescribed to patients to lower the cholesterol levels and aspirin is prescribed because of the anti-coagulative effects. Both medications resulted in a decline in the incidence of cardiovascular disease (CVD) during the last decade, but the fact that CVD is still the leading cause of death in the Western world implies that new therapies that regulate the immune response in atherosclerosis may be able to substantially reduce the incidence of CVD. This forms the reason why a substantial effort is put into the development of immunotherapies for atherosclerosis.
One of the strategies described in this thesis is vaccination using oxLDL-loaded DCs. This technique has some benefits when compared with existing vaccination/immunization protocols against oxLDL. DC-vaccination as used in **chapter 4** was very efficient, reducing atherosclerosis with more than 85%, and no adjuvant is needed to activate the immune system, which will reduce the time needed to optimize the vaccination protocol. When immunizing mice with modified LDL, an adjuvant is needed and the reduction in atherosclerosis was less prominent than in the DC vaccination study. An adjuvant gives an extra boost of the immune system and more specific strategies without an adjuvant are supposed to lead to less side effects. Therefore, the use of DCs as a vaccination unit is considered to be promising. At the moment, DC vaccination therapies are tested in clinical trials to treat several types of tumors. A complication of this technique is however that the DC production is labor-intensive and expensive, requiring specialized and costly manufacturing facilities.

A second strategy used in this thesis is the induction of oral tolerance and activation of Tregs. Tregs are increasingly used as therapeutic intervention method. In several diseases the number or function of Tregs is decreased and restoring the normal numbers may be protective. Adoptive transfer of Tregs could be successful but no clinical trials in humans have been reported yet. A major problem in this strategy is to obtain large numbers of these cells as per patient a high number of cells will be needed. Another method to induce Tregs is oral tolerance induction. Oral tolerance has been used in mouse models to treat several autoimmune diseases and it is currently tested in several clinical trials to treat type 1 diabetes mellitus, allergy, arthritis, multiple sclerosis etc. In **chapter 2 and 3** oral tolerance was induced to two important auto-antigens in atherosclerosis; HSP60 and oxLDL. The results of the clinical trials on other autoimmune disorders are very diverse. Some are successful, while others have no effect. Most of the clinical trials to treat several autoimmune disorders that are performed nowadays have however one common problem, which is the fact that the administered antigens are non-self molecules. Examples are clinical trials with myelin in patients with multiple sclerosis and collagen in patients with arthritis. In this thesis, oxLDL is used, which can be generated via oxidation of the patient's own LDL. This will be an advantage in clinical trials, because less side effects will be induced. We now intend to test oral tolerance to oxLDL in a clinical trial.

The third strategy used as an immunotherapy in this thesis was the activation of NKT cells. α-GalCer and OCH are both strong activators of NKT cells with protective effects in mouse models for several autoimmune diseases. Because α-GalCer had anti-tumor effects in mice, α-GalCer as used in **chapter 6** is tested in clinical trials to treat several types of cancer. In **chapter 5** DC-vaccination was combined with NKT cell activation via loading of OCH on the DCs. NKT cell activation via α-GalCer loaded DCs is also tested in clinical trials to treat lung cancer and it is shown that this activates NKT cells very efficiently. The only problem however is that the natural activator of NKT cells in atherosclerosis is not known. The discovery of this ligand will be a breakthrough in atherosclerosis research and will result in more opportunities for therapies in atherosclerosis. In **chapter 7** it is observed that oxLDL may be one of these ligands but it needs further investigation to what extent this oxLDL is responsible for activation of NKT cells in atherosclerosis.

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Nederlandse samenvatting

Hart- en vaatziekten zijn ondanks het vele onderzoek en de verschillende cholesterol en bloeddruk verlagende middelen nog steeds doodsoorzaak nummer 1 in Nederland. 33% van alle sterfgevallen in 2006 waren direct of indirect gerelateerd aan een hart- of een vaat-aandoening met als belangrijkste gevolg een beroerte of een acuut hartinfarct. Hart- en vaatziekten worden voornamelijk veroorzaakt door aderverkalking (atherosclerose). Zoals bij de meeste mensen wel bekend is, dragen veel factoren bij aan het ontstaan van hart- en vaatziekten, zoals hoge cholesterol waarden in het bloed, een verhoogde bloeddruk, roken, te weinig lichaamsbeweging en suikerziekte (diabetes). De aderverkalking uit zich als een verdikking van de bloedvatwand en wordt verkalking genoemd omdat in een later stadium veel calcium in de verdikking zit die leidt tot de vernauwing van een slagader. Dit proces begint al op vroege leeftijd en vlak na de puberteit zijn de eerste verdikkingen waar te nemen in verschillende slagaderen. Verwarrend is daarom de naam aderverkalking, want atherosclerose vind alleen plaats in de slagaderen. Wat veel mensen echter niet weten is dat atherosclerose in feite een autoimmuun component bevat waarbij slechte voeding, roken en weinig lichaamsbeweging ook via het immuun system de progressie van atherosclerose kunnen beinvloeden.

Ontstaan van atherosclerose; een grote rol voor het immuun systeem

Het begin van atherosclerose is een ophoping van vet en met name cholesterol in daarvoor gevoelige plaatsen in de vaatwand van de slagaderen. Deze plaatsen hebben vaak een turbulente bloedstroom waardoor het endotheel dat zorgt voor de bescherming van de vaatwand doorlaatbaar wordt voor vetten en bloedcellen. Het endotheel kan gevoeliger zijn voor de turbulente bloedstroom door bijvoorbeeld roken, maar ook door de hoeveelheid vet in het bloed. Het beschadigde endotheel zorgt vervolgens voor het aantrekken van allerlei ontstekingscellen, de witte bloedcellen. Monocyten en T cellen, twee soorten witte bloedcellen, migreren in eerste instantie via het endotheel de vaatwand in. Monocyten differentieren in de vaatwand tot macrofagen en kunnen het opgehoopte vet in de vaatwand opnemen. Dit leidt tot de vorming van een zogenaamde 'foamcell' of schuim cel. De T cellen die worden aangetrokken in de vaatwand kunnen daar opnieuw geactiveerd worden doordat ze specifieke moleculen, antigenen, herkennen in de verdikking die nu een plaque genoemd wordt. Deze antigenen worden gepresenteerd door antigeen presenterende cellen zoals de macrofagen en dendritische cellen (DCs). Omdat er bij atherosclerose sprake is van een autoimmuun reactie betekent dit dat een immunologische reactie wordt opgewekt tegen lichaamseigen antigenen. De antigenen kunnen bijvoorbeeld immunogeen worden doordat ze een structurele verandering ondergaan. Dit is het geval bij lage-dichtheid lipoproteinen (LDL). In de vaatwand kan dit niet-

immunogene LDL geoxideerd worden tot oxLDL. Deze gemodificeerde variant kan opgenomen worden door macrofagen en DCs en via MHC moleculen gepresenteerd worden aan T cellen. Naast de modificatie van lichaamseigen stoffen die zo immunogeen worden, zijn er ook moleculen die immunogeen worden doordat ze grote structurele overeenkomsten hebben met moleculen die op bacteriën tot expressie komen. De "heat shock proteïnen" zijn hier het bekendste voorbeeld van en ook zij vormen een belangrijke groep van auto-antigenen in atherosclerose.
De T cellen die dit antigeen herkennen raken geactiveerd en produceren cytokines. Deze cytokines zijn kleine moleculen die een grote invloed kunnen hebben op het verdere verloop van een ontsteking. Over het algemeen zijn de T cellen in atherosclerose Th1 cellen en produceren ze atherosclerose-bevorderende cytokines (Th1 cytokines). Tegenover Th1 cytokines staan de Th2 cytokines die geproduceerd worden door onder andere Th2 cellen. Deze cytokines hebben over het algemeen een positieve uitwerking op atherosclerose. Lang is er verondersteld dat een verstoring in de balans van deze Th1 en Th2 cellen aan de basis lag van de immunologische reactie in atherosclerose.

Regulatoire T cellen

Uit recent onderzoek is gebleken dat niet alleen de verstoorde balans tussen deze Th1 en Th2 cellen een belangrijke veroorzaker is van de schadelijke immunologische reactie in atherosclerose. Een verstoorde balans tussen pathogene T cellen (Th1 en Th2) aan de ene kant en regulatoire T cellen (Tregs) aan de andere kant vormt een nieuwe hypothese. Tregs zijn T cellen die gekenmerkt worden door de productie van atherosclerose-remmende cytokines als IL-10 en TGF-β. Natuurlijke Tregs zijn te herkennen aan de specifieke expressie van Foxp3 dat een rol speelt in de ontwikkeling van de Tregs. Naast de natuurlijke Tregs, zijn er ook adaptieve Tregs die na activatie Foxp3 tot expressie brengen (Th3 cellen). Deze Th3 cellen produceren voornamelijk TGF-β. In de **hoofdstukken 2 en 3** is gebruik gemaakt van een methode waarbij specifieke regulatoire T cellen kunnen worden geïnduceerd. Deze methode, orale tolerantie inductie, is een eenvoudige methode waarbij een antigeen, waartegen een regulatoire T cel response opgewekt dient te worden, toegediend wordt via de mond. Deze route zorgt ervoor dat het antigeen in de darmen komt. Daar wordt het door gespecialiseerde cellen opgenomen en uiteindelijk wordt het antigeen door tolerogene dendritische cellen gepresenteerd. Afhankelijk van de toegediende dosis kan het lokale cytokine milieu in de darmen zorgen voor een activatie van Tregs. In **hoofdstuk 2** werd aangetoond dat de orale toediening van oxLDL leidde tot een 71% reductie in initiële atherosclerotische plaque vorming. In een tweede experiment is aangetoond dat orale tolerantie inductie tegen oxLDL ook een remmend effect heeft op al aanwezige atherosclerose. De tolerantie inductie tegen oxLDL leidde ook tot een toename in $CD4^+CD25^+Foxp3^+$ Tregs in lymfeknopen en de milt. Miltcellen, geïsoleerd uit met oxLDL behandelde muizen, produceerden meer TGF-β dan miltcellen uit controle behandelde muizen. Dit wijst op een activatie van oxLDL-specifieke Tregs. Ook leidde de tolerantie inductie tot een verhoogde expressie van genen specifiek voor Tregs,

namelijk Foxp3, CD25 en CTLA-4 in de atherosclerotische plaque.
Dezelfde techniek is gebruikt in **hoofdstuk 3**. Hier is echter gebruik gemaakt van HSP60 en een van HSP60 afgeleid peptide (HSP60(253-268)). Orale toediening van beide antigenen leidde tot een reductie in initiële plaque vorming (81%). In de met HSP60 behandelde muizen werd 4 dagen na de behandeling een toename in het aantal $CD4^+CD25^+Foxp3^+$ Tregs waargenomen in het bloed en de Peyer's patches in de darmen. Na 2 weken was het aantal Tregs ook toegenomen in de mesenterische lymfeknopen en de milt terwijl het aantal in de Peyer's patches weer was afgenomen. Dit kan verklaard worden door de migratie van Tregs van de Peyer's patches via het bloed naar de lymfeknopen en de milt. In deze studie bleken de specifieke cellen uit de mesenterische lymfeknopen van HSP60 behandelde muizen in response op HSP60 niet alleen meer TGF-β te produceren maar ook meer IL-10 dan cellen uit een controle behandelde muis.
Samengevat blijken de specifieke $CD4^+CD25^+Foxp3^+$ Tregs een zeer positieve uitwerking te hebben op atherosclerose en beide studies ondersteunen dan ook de nieuwe kijk op de verstoorde balans tussen verschillende T cellen in atherosclerose. Het gebruik van orale tolerantie vormt dus een succesvolle methode om de immunologische reactie in atherosclerose ten gunste te keren.

Dendritische cellen

Dendritische cellen zijn belangrijke antigeen presenterende cellen en spelen een rol in de activatie van T cellen en ook natuurlijke killer T (NKT) cellen die later in het proefschrift behandeld zullen worden. Het feit dat ze zo potent zijn in het activeren van deze cellen maakt ze uitermate geschikt om ze bij vaccinatie te gebruiken. In verschillende studies was al beschreven hoe DCs kunnen worden beladen met antigenen en na teruggave aan de muizen een beschermend effect kunnen uitoefenen. De techniek van het toedienen van antigenen door ze toe te dienen via DCs die opgeladen zijn met het antigeen werd gebruikt in **hoofdstuk 4**. In deze studie werden DCs geladen met oxLDL en intraveneus toegediend aan LDLr deficiënte muizen alvorens atherosclerose werd geïnduceerd door een vetrijk dieet. Deze geladen DCs leidden tot een 87% tot 92% reductie in atherosclerose. Daarbij waren de laesies in met oxLDL geladen DCs behandelde muizen stabieler dan in de controle muizen. De stabielere plaques hebben minder kans om tot een ruptuur te leiden, mogelijk gevolgd door een stolling en afsluiting van het bloedvat. Een verklaring voor de reductie in plaquegrootte werd gevonden in het feit dat er een verhoogde concentratie van IgG antilichamen tegen oxLDL werd waargenomen in het serum van de muizen die met oxLDL geladen DCs zijn behandeld. Dit was toe te schrijven aan een activatie van oxLDL specifieke T cellen. Het serum van de behandelde muizen bleek ook een remmende werking te hebben op de vorming van met vet beladen macrofagen, de zogenaamde schuimcellen. Dit kan veroorzaakt worden door verschillende mechanismen. IgG antilichamen kunnen complexen vormen met oxLDL waardoor dit oxLDL sneller uit de circulatie wordt verwijderd, nog voordat het enig negatief effect kan hebben. Een ander verklaring zou kunnen zijn dat door de complex vorming, het oxLDL minder goed via scavenger receptoren opgenomen kan worden en daardoor minder vetstapeling geeft. De

exacte verklaring is echter nog onduidelijk en meer experimenten zullen hier uitsluitsel over geven.

NKT cellen

Naast de Th1/Th2 cellen en Tregs is uit recent onderzoek gebleken dat ook NKT cellen een belangrijke rol spelen in atherosclerose. NKT cellen zijn Natural Killer cellen met T cel eigenschappen. De normale NK cellen zijn verantwoordelijk voor het aspecifiek verwijderen van lichaamsvreemde substanties terwijl NKT cellen een specifieke T cel receptor hebben waardoor ze alleen op bepaalde antigenen reageren. In tegenstelling tot de normale T cellen worden NKT cellen echter niet geactiveerd door eiwit-antigenen maar door vet (lipide)-antigenen. De activatie van NKT cellen leidt tot de productie van verschillende cytokines, zowel pro-atherogeen (Th1) als anti-atherogeen (Th2). Dit geheel, en met name de response op lipide-antigenen, maakt deze cellen extra interessant in het proces van atherosclerose. Uit een aantal recente publicaties is gebleken dat het uitschakelen van NKT cellen in muizen leidt tot een vermindering van atherosclerose. Daarnaast is het mogelijk om specifiek NKT cellen te activeren met een synthetisch ligand, het zogeheten α-galactosylceramide (α-GalCer). Naast α-GalCer is er op dit moment ook nog een tweede synthetisch ligand, OCH. OCH leidt net als α-GalCer tot activatie van de NKT cellen, maar in tegenstelling tot α-GalCer leidt dit tot de productie van meer anti-atherogene cytokines; voornamelijk IL-10.
In **hoofdstuk 5 en 6** is voor het eerst aagetoond dat NKT cellen ook een beschermende rol kunnen spelen in atherosclerose. In **hoofdstuk 5** werden LDL receptor (LDLr) en apolipoproteine E (apoE) deficiënte muizen na de inductie van atherosclerose door een vetrijk dieet, gedurende 7 weken twee maal per week behandeld met α-GalCer. Dit leidde tot een 84% afname in plaque vorming in de LDLr deficiënte muizen. Er was echter geen significante afname van atherosclerose in apoE deficiënte muizen. De verschillen tussen beide muismodellen werden verder benadrukt door de verschillen in proliferatie en cytokine profiel na *in vitro* stimulatie van miltcellen met α-GalCer. LDLr deficiënte miltcellen prolifereerden veel sterker in response op α-GalCer dan apoE deficiënte miltcellen, terwijl de toename in IL-4 en IL-10 productie na α-GalCer stimulatie in LDLr deficiënte muizen ook veel groter was. Ook resulteerde het vetrijke dieet in LDLr deficiënte muizen tot een toename in het percentage NKT cellen in de milt en de lever. Dit effect was afwezig in de eerste weken in de apoE deficiënte muizen, maar later na 4.5 week dieet werd ook in deze muizen een toename in NKT cellen in de lever waargenomen. Uit andere studies was al gebleken dat de behandeling van apoE deficiënte muizen met α-GalCer niet bevorderend was voor atherosclerose. Een verklaring voor de verschillen in response op α-GalCer tussen beide muismodellen kan worden gevonden in het feit dat apoE een belangrijke rol speelt in de presentatie van lipide antigenen. ApoE deficiënte muizen missen apoE en hebben daardoor een minder effectieve lipide antigen presentatie. Daarnaast lijkt het toedienen van een vetrijk dieet de NKT cellen in LDL deficiënte muizen al te activeren waarna α-GalCer een positieve uitwerking heeft en een toename in Th2 cytokines

veroorzaakt. Mede daardoor veroorzaakt dit een vermindering in plaque grootte. In apoE deficiënte muizen is deze activatie via het dieet afwezig op het moment dat α-GalCer wordt toegediend. We veronderstellen nu dat de latere effecten van het dieet in combinatie met α-GalCer resulteren in het feit dat er geen verschil in plaque grootte wordt waargenomen, terwijl in andere studies zonder een vetrijk dieet een enorme toename in plaque grootte in deze muizen werd beschreven.

In **hoofdstuk 6** is aangetoond dat NKT cellen dusdanig gestimuleerd kunnen worden zodat ze een positieve uitwerking hebben op de immunologische reactie tijdens atherosclerose. In dit hoofdstuk werd gebruik gemaakt van de α-GalCer analoog OCH. Het is bekend dat NKT cellen, geactiveerd door dit ligand, meer Th2 cytokines produceren. In een eerdere publicatie was gevonden dat dit ligand echter ook een negatieve werking had op atherosclerose geïnduceerd in apoE deficiënte muizen. In onze studie is dit bestudeerd maar dan in LDLr deficiënte muizen. In een eerste experiment werden LDLr deficiënte muizen waarin atherosclerose was geïnduceerd gedurende 7 weken twee maal per week behandeld met OCH door middel van intra-peritoneale injecties. Dit bleek echter niet succesvol. Daarop werd overgestapt op een ander vorm van toediening van OCH, namelijk geladen op DCs. Uit de literatuur bleek namelijk dat dit een effectieve manier kan zijn om een langdurige NKT cel activatie te induceren. DCs migreerden na intraveneus te zijn geinjecteerd voornamelijk naar de longen en de lever, maar ook naar de milt. Migratie naar de lever is belangrijk aangezien daar de meeste NKT cellen aanwezig zijn. De behandeling van LDLr deficiënte muizen met OCH geladen DCs, nog voordat atherosclerose werd geïnduceerd, leidde tot een significante 58 tot 71% reductie in plaque vorming. De behandeling ging gepaard met een toename in NKT cellen in zowel het bloed als de lever. De NKT cellen bleken in response op OCH geladen DCs ook meer IL-10 te produceren dan NKT cellen uit muizen behandeld met controle DCs. De toegenomen productie van IL-10, met name in de lever, kan de oorzaak zijn van de reductie in cholesterol die werd waargenomen in muizen behandeld met OCH-geladen DCs aan het eind van het experiment.

NKT cel liganden

In **hoofdstuk 7** is getracht om de natuurlijke ligand voor de hierboven genoemde NKT cellen te achterhalen. Aangezien NKT cellen geactiveerd worden door lipide antigenen en er in atherosclerose veel lipide antigenen aanwezig zijn, werd het mogelijk geacht dat een van deze lipiden NKT cel activatie tot gevolg heeft. Deze gedachte werd ondersteund door het feit dat afwezigheid van CD1d een remmend effect had op atherosclerose. CD1d is een receptor voor lipide antigenen. In deze studie is net als in **hoofdstuk 5** aangetoond dat een vetrijk dieet een stimulerende werking heeft op NKT cellen. LDLr deficiënte muizen welke het vetrijke dieet hebben gegeten bezitten veel meer NKT cellen in de lever, de milt en lymfeknopen dan wanneer er geen vetrijk dieet wordt gegeten. Er is ook aangetoond dat oxLDL tot minder proliferatie van miltcellen leidt wanneer NKT cellen afwezig zijn. Het kruisen van LDLr deficiënte muizen met Jα281 deficiente muizen (missen NKT cellen) had echter verassend genoeg geen

effect op de plaque grootte. Dit komt overigens overeen met een aantal eerdere studies, waarin CD1d deficiëntie alleen een vermindering in atherosclerose veroorzaakte in de initiële fase en niet beschermend voor meer gevorderde vaatvernauwing.

Samenvattend laat dit proefschrift zien dat de activiteit van verschillende soorten T en NKT cellen tijdens atherosclerose op verschillende manieren therapeutisch te beïnvloeden is. Regulatoire T cellen blijken een uitermate positieve uitwerking te hebben op atherosclerose. Daarnaast blijkt dat met behulp van DCs het activeren van oxLDL-specifieke T cellen en de daarop volgende toename in oxLDL specifieke antilichamen ook een positief effect heeft op atherosclerose. Verder is aangetoond dat NKT cellen een complexe rol spelen in de immunologie van atherosclerose. Ze kunnen een beschermende rol hebben in atherosclerose, wanneer ze 1) direct geactiveerd worden met behulp van α-GalCer, of wanneer ze 2) geactiveerd worden met een ligand (OCH) geladen op DCs. De studies in dit proefschrift leiden niet alleen tot meer inzicht in de rol van het immuunsysteem bij de vet-geïnduceerde aderverkalking, maar creëren een therapeutisch protocol dat in de toekomst mogelijk kan worden toegepast ter vermindering van de incidentie van hart- en vaatziekten bij de mens.

Abbreviations

β2GPI	β2-glycoprotein I
Ab	antibody
APC	antigen presenting cell
apoE/apoB	apolipoprotein E/apolipoprotein B
CC/CXC	chemokine
CCL/CXCL	chemokine ligand
CD	cluster of differentiation
CIA	collagen induced arthritis
CMV	Cytomegalovirus
conA	concanavalin A
CTLA	cytotoxic T-lymphocyte antigen
CVD	cardiovascular disease
DC	dendritic cell
EAE	experimental acquired encephalomyelitis
FACS	fluorescent-activated cell sorting
Foxp3	forkhead box p3
GalCer	galactosylceramide
GFP	green-fluorescent protein
HDL	high-density lipoprotein
HSP	heat shock protein
i.p.	intraperitoneal
i.v.	intravenous
ICAM-1	intercellular adhesion molecule-1
IDL	intermediate-density lipoprotein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
imDC	immature dendritic cell
LDL	low-density lipoprotein
LDLr	low-density lipoprotein receptor
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein
M-CSF	macrophage colony stimulating factor
MDA-LDL	malondialdehyde modified LDL
mDC	mature dendritic cell
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NF-κB	nuclear factor κB
NK	natural killer
NKT	cell natural killer T cell
oxLDL	oxidized low-density lipoprotein

PBS	phophate buffered saline
PC	phosphatidyl choline
PE	phosphatidyl ethanolamin
s.i.	stimulation index
SMC	smooth muscle cell
SR	scavenger receptor
STI	soybean trypsin inhibitor
TβRII	transforming growth factor β receptor II
TCR	T cell receptor
TGF	transforming growth factor
Th1/Th2	T helper 1/T helper 2
TLR	Toll-like receptor
TNF	tumor necrosis factor
Tr1	regulatory T cell type 1
Treg	regulatory T cell
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein

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Curriculum Vitae

Gijs van Puijvelde werd geboren op 6 oktober 1978 in Terneuzen. In mei 1997 werd het VWO diploma behaald aan de Stedelijke Scholengemeenschap "De Rede" te Terneuzen. In datzelfde jaar werd begonnen met de studie Medische Biologie aan de Vrije Universiteit van Amsterdam. Het propaedeutisch examen werd in augustus 1998 gehaald. Van januari tot en met september 2000 werd in het kader van de hoofdvakstage onderzoek verricht binnen de vakgroep CelBiologie onder leiding van drs. S. Floris en dr. E. de Vries met als onderwerp 'Expression of CS-1 sequence by astrocytes and its role in the adhesion of monocytes'. Van november 2000 tot en met augustus 2001 werd een tweede stage gevolgd bij de vakgroep Neurobiologie aan het Swammerdam institute for Life Sciences, onderdeel van de Universiteit van Amsterdam, onder leiding van drs. D. Marsen en Prof.dr. W. Wadman. Dit onderzoek was getiteld "The effects of 5-MOP on two types of voltage-gated K^+ currents: the A- and D-current." Het doctoraal examen werd in januari 2002 gehaald.
Van februari 2002 tot augustus 2006 werd als assistent in opleiding het in dit proefschrift beschreven onderzoek uitgevoerd bij de vakgroep Biorfarmacie van het Leiden/Amsterdam Center for Drug Research onder leiding van dr. J Kuiper en Prof. dr. Th.J.C. van Berkel. Dit onderzoek maakte deel uit van een door de Nederlandse Hartstichting gefinancieerd project. Tijdens zijn periode als assistent in opleiding heeft hij in januari 2003 een prijs gekregen voor de beste posterpresentatie tijdens "The 5th St. Gerlach Vascular Biology Workshop." In april 2004 ontving hij een minifellowship tijdens het 7e symposium van de "Dutch Atherosclerosis Society". Daarnaast onving hij in april 2004 een prijs voor de beste poster presentatie tijdens het "LACDR Spring Symposium". Van oktober 2006 tot april 2007 werd er vervolgonderzoek verricht aan het Karolinska instituut in Stockholm, Zweden. Dit met als doel het opzetten van een langdurig samenwerkingsverband tussen de afdeling Biofarmacie in Leiden en de afdeling "Experimental Cardiovascular Reasearch" in Stockholm. Sinds 15 mei 2007 is hij aangesteld als Post-doc binnen de vakgroep Biofarmacie van het Leiden/Amsterdam Center for Drug Research op een project van Top Instituut Pharma.

Nawoord

Op moment van schrijven van het nawoord zit ik op het vliegveld van Stockholm. Zes maanden heb ik daar onderzoek gedaan en ook een groot deel van mijn proefschrift heb ik daar tijdens de koude en vooral donkere, depressieve winter geschreven. Gedurende de afgelopen jaren als AIO op de afdeling Biofarmacie onder begeleiding van Johan Kuiper en Theo van Berkel, heb ik gewerkt, genoten, gelachen, gebaald, en de laatste periode ook geploeterd. Maar, zoals elke Zeeuw weet, Luctor et Emergo, "ik worstel en kom boven" en het lijkt er nu op dat dat toch wel gelukt is. Natuurlijk is dat allemaal niet vanzelf gegaan en hebben veel mensen mij hierbij gesteund. Vooral tijdens mijn soms wel lastige periode in Stockholm zal ik de hulp en mentale steun nooit vergeten. Het lastige van een dankwoord is altijd bij wie je dan moet beginnen. In dit geval moet ik dan echt beginnen bij kamer 839. We hebben er een gezellig hok van gemaakt, soms misschien iets te gezellig (cafe 839) maar elke ochtend kwam ik met een lach op het gezicht de kamer binnen en vaak ging ik er ook met een lach weer uit. Arnaud, jij hebt me op gang getrokken en ik denk dat het aardig gelukt is. Ik zal de congressen, het fietsen, tafeltennis en het pinguinen nooit vergeten. Filip, alias Fluffie, de gezellige woensdagavonden moeten we snel weer terug oppakken want een Jupke gaat er altijd in. Jou gezelligheid en steun maken je tot een perfecte paranimf. Laten we hopen dat we die tafel bij mij nog eens kunnen testen. Ruud, kameroudste en nog altijd anti-Zeeuw, nu ik nog wat langer blijf zal je toch echt aan het Zeeuws moeten wennen. Eva, ik zal onze samenwerking en met name de uren achter de FACS nooit vergeten. Daarnaast zal ik je gezellige aanwezigheid bij Biofarmacie enorm missen. Kim, we hebben een leuke succesvolle samenwerking achter de rug en laten we dat nog maar even doorzetten, al ben ik als tolk Vlaams-Nederlands inmiddels wel overbodig. Illiana, het nieuwste 839 lid, we moeten het maandelijkse "Grieken" zeker in stand houden, want dat zijn altijd hele gezellige avonden.
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De dierenverzorgsters in Zweden zien er dan wel een stuk beter uit, toch mag

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Gijs van Puijvelde
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